XVI. SOME EFFECTS OF PYROPHOSPHATE ON THE METABOLISM OF TISSUES

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(*Received 24 November 1938*)

Pyrophosphate has been shown by Dixon & Elliott [1929] to inhibit the respiration of minced tissue. For liver mince the inhibition was less than with cyanide, and small concentrations produced a slight acceleration. With muscle, on the other hand, pyrophosphate produced a greater inhibition than cyanide, $M/30$ pyrophosphate causing more than 80% inhibition. According to Keilin [1929] pyrosphophate has no effect on indophenol oxidase (cytochrome oxidase). Dixon & Thurlow (quoted by Dixon & Elliott [1929]) found that succinic dehydrogenase is definitely inhibited by $M/1000$ pyrophosphate while xanthine dehydrogenase is unaffected by $M/25$. More recently, Leloir & Dixon [1937] have made a systematic survey of different dehydrogenases and have found that, of the nine dehydrogenases studied, succinic is the only one which is inhibited by pyrophosphate, so that pyrophosphate probably acts as a respiratory inhibitor by specific inhibition of this enzyme.

Succinic dehydrogenase has come into prominence recently with Szent-Györgyi's theory of tissue respiration [1937] in which succinic, fumaric, malic and oxaloacetic acids, with their appropriate enzyme systems, can act as hydrogen transportases. Succinate is the last member of the chain of reactions and from this H is transferred to molecular O_2 through cytochrome and cytochrome oxidase. If the C_4 dicarboxylic system accounts for the bulk of tissue respiration one would expect respiration to fall off when succinic dehydrogenase is inhibited, the extent, of course, depending on whether or not the concentration of succinic dehydrogenase is the limiting factor. This was indeed shown to be the case by Annau [1935], Gözsy & Szent-Györgyi [1934], Greville [1936], and Stare [1936] who found that malonate inhibited the respiration of various tissues. Boyland & Boyland [1936] reported an inhibition in respiration of tumour tissue by the addition of malonate. Elliott & Greig [1937], using Ringer-Krebs medium, found that malonate inhibited the respiration of liver slices about 15% and that of kidney slices by 50–60%, presumably by specific inhibition of succinic dehydrogenase.

Johnson [1936] found that the rate of aerobic removal of Na α-glycerophosphate by normal minced brain tissue is increased by added Na pyrophosphate. He considers this effect similar to that of adenyl pyrophosphate acting as coenzyme for yeast glycerophosphate dehydrogenase as shown by Lehmann [1934].

Pyrophosphate seems to exert a stimulating effect on certain other enzymes. Green [1936], working on isolated enzyme systems, found that pyrophosphate has no effect on lactic dehydrogenase but causes 100% acceleration of malic dehydrogenase.

Peters & Sinclair [1933, 1], using minced pigeon brain in phosphate buffer, found that Na pyrophosphate added alone had little influence upon maintenance of respiration in the surviving tissue but that, added with lactate, it increased the respiration rate considerably. Respiration was also maintained at a more constant rate over a period of 3 hr. instead of falling off rapidly as was the case with added lactate alone. They found that the improvement in respiration by pyrophosphate with added lactate was, in part, due to an increase in fluoride-sensitive respiration, and suggested that the hexosediphosphate (or triose-phosphate) system is in some way involved in the metabolism of lactate. The optimum concentration of pyrophosphate was 0·2 % which is of the same order as that possible in the cell. Peters considered that these substances (lactate, pyrophosphate and probably glycerophosphate) may be present in the tissue and cause the initial high rate of respiration, but diffuse out of the cell and gradually become ineffective.

In avitaminous brain Peters *et al.* [1933, 2; 1935] found that pyrophosphate ($M/100$) enhanced the catatorulin effect of vitamin B_1 in the metabolism of lactate and pyruvate. It may be that cocarboxylase is synthesized in this tissue from vitamin B_1 and pyrophosphate.

It thus appears that pyrophosphate has several possible effects on the respiration mechanisms of minced tissues. It can increase oxidations by a stimulating effect on malic or α-glycerophosphate dehydrogenations and it can increase lactate oxidation and the catatorulin effect of vitamin B_1, but it can also inhibit respiration through its effect on succinic dehydrogenase. The results of a survey of the effects of pyrophosphate on respiring slices of different tissues are presented here.

Experimental

The methods employed were those described by Elliott and co-workers [1934, 1935] for the Dixon-Keilin differential manometer. When glucose was present in the medium its concentration was 0·24 %. The concentrations of added substrates were: *dl*-lactate $M/25$; pyruvate, succinate and *l*-malate $M/50$. The concentration of pyrophosphate varied as shown in the table. The solution, freshly prepared for each experiment, was brought to *p*H 7·4 by addition of dilute HCl [see Leloir & Dixon, 1937]. The experimental period was 90 min. in all cases.

The rat tissues were prepared as described by Elliott *et al.* Ox eyes were obtained from the slaughter house on the morning of killing and the retina was removed as will be described in a later paper. With the rat tissues the results were calculated on the final dry wt. [see Elliott *et al.* 1937]. With retina the tissue disintegrated to such an extent, especially after the addition of acid, that final dry wts. were only approximate. The results were therefore calculated on the initial dry wts. obtained from the wet wt. of tissue taken and the dry wt./wet wt. ratio determined on separate samples.

The terms used for expressing results are those defined by Elliott *et al.* Variations of 10 % are considered to be within the experimental error and when the apparent effect of pyrophosphate is within this range the % effect is not given in Table I. When the values obtained are small, as is often the case for Q_A, the effect of pyrophosphate may cause a large % change but the values are too small for accurate experimental measurement. In these cases the % change is not given but when there is a definite acceleration or inhibition it is designated by a plus or a minus sign in Table I.

Table I

Tissue	Addition	Final conc. of pyrophosphate	Glucose	$-Q_{O_2}$	R.Q.	Q_A	% effect of pyrophosphate on Q_{O_2}	% effect of pyrophosphate on Q_A
Rat tumour Walker 256	None	–	+	9·2	1·0	+20·2		
	Pyrophosphate	*M*/100	+	10·5	0·78	+19·0		
	None	–	+	10·1	0·98	+18·7		
	Pyrophosphate	*M*/30	+	6·5	0·65	+17·7	−35	
	None	–	–	11·0	0·68	+ 0·9		
	Pyrophosphate	*M*/30	–	10·1	0·69	+ 1·4		
	Lactate	–	–	11·9	0·79	− 0·5		
	Lactate + pyrophosphate	*M*/50	–	10·5	0·76	+ 1·1	−12	+
Rat testis	None	–	–	6·9	0·68	+ 1·3		
	Pyrophosphate	*M*/30	–	4·8	0·64	+ 2·2	−30	
	None	–	+	11·5	0·94	+ 6·9		
	Pyrophosphate	*M*/30	+	9·9	0·83	+ 5·0	−14	–
	Lactate	–	–	16·3	0·86	− 2·8		
	Lactate + pyrophosphate	*M*/50	–	11·9	0·83	− 1·1	−27	–
Rat liver	None	–	–*	12·8	0·85	+ 0·1		
	Pyrophosphate	*M*/30	–	11·6	0·76	+ 0·4	−10	
	Pyrophosphate	*M*/30	+	8·8	0·81	+ 2·6	−31	+
	Lactate	–	–	12·5	0·81	− 3·3		
	Lactate + pyrophosphate	*M*/30	–	9·5	0·88	− 0·3	−24	
	None	–	–	12·5	0·75	+ 1·3		
	Pyrophosphate	*M*/30	+	10·6	0·72	+ 1·8	−16	
	Pyrophosphate	*M*/30	+	10·0	0·85	+ 1·8	−20	
	Lactate	–	–	13·7	0·89	− 4·5		
	Lactate + pyrophosphate	*M*/30	–	12·7	0·88	− 0·6		−88
Rat brain	None	–	+	16·7	0·93	+ 5·8		
	Pyrophosphate	*M*/50	+	15·7	0·96	+ 7·4		
	Lactate	–	–	11·9	0·95	− 2·8		
	Lactate + pyrophosphate	*M*/50	–	12·1	0·94	− 2·2		
Rat kidney	None	–	+	25·9	0·90	− 1·1		
	Pyrophosphate	*M*/100	+	27·1	0·79	+ 2·8		+
	None	–	–	24·4	0·75	+ 0·6		
	Pyrophosphate	*M*/50	–	22·7	0·74	+ 3·6		+
	None	–	+	27·7	0·84	+ 0·2		
	Pyrophosphate	*M*/50	+	31·4	0·82	+ 3·1	+13	+
	None	–	+	25·9	0·80	+ 0·4		
	Pyrophosphate	*M*/30	+	30·2	0·78	+ 3·6	+17	+
	Pyrophosphate	*M*/100	+	30·7	0·83	+ 2·1	+18	+
	Pyrophosphate	*M*/1000	+	26·8	0·89	+ 1·6		+
	None	–	+	25·1	0·85	− 0·9		
	NaCl 4*M*/30	–	+	26·3	0·82	+ 1·6		+
	None	–	–	19·6	0·79	− 1·4		
	NaCl 4*M*/30	–	–	17·8	0·69	+ 0·4		
	Succinate	–	–	46·7	0·74	−18·2		
	Succinate + pyrophosphate	*M*/50	–	40·4	0·79	− 7·1	−12	−61
	Malate	–	–	34·3	1·18	−17·1		
	Malate + pyrophosphate	*M*/50	–	29·7	0·95	− 3·7	−13	−78
	Pyruvate	–	–	37·8	1·23	−20·2		
	Pyruvate + pyrophosphate	–	–	36·2	1·08	− 7·3		−60
	Lactate	–	–	27·2	0·81	− 6·5		
	Lactate + pyrophosphate	*M*/50	–	27·0	0·83	− 2·3		−65

* The presence or absence of glucose does not affect the normal respiration of liver [see e.g. Elliott *et al.* 1937].

Table I (*cont.*)

Tissue	Addition	Final conc. of pyrophosphate	Glucose	$-Q_{O_2}$	R.Q.	Q_A	% effect of pyrophosphate Q_{O_2}	% effect of pyrophosphate Q_A
Ox retina	None	–	+	9·8	0·86	+ 8·4		
	Pyrophosphate	*M*/50	+	15·2	0·98	+ 9·9	+55	
	None	–	–	7·3	0·99	– 0·9		
	Pyrophosphate	*M*/50	–	9·3	1·00	– 0·8	+27	
	Lactate	–	–	11·1	0·90	– 2·3		
	Lactate + pyrophosphate	*M*/50	–	14·5	0·97	– 3·1	+31	+
	None	–	–	6·4	0·93	– 1·0		
	Pyrophosphate	*M*/50	–	7·1	0·89	– 0·3	+	
	Lactate	–	–	11·1	0·85	– 2·3		
	Lactate + pyrophosphate	*M*/50	–	16·2	0·95	– 2·9	+46	+
	None	–	+	11·6	0·88	+ 7·9		
	Pyrophosphate	*M*/50	+	15·2	0·91	+ 9·3	+31	+18

Discussion

The effect of pyrophosphate on tissue slices varies with the concentration of pyrophosphate, the tissue used and the substrate being metabolized. With rat tumour (Walker 256), dilute pyrophosphate (*M*/100) in the presence of glucose increases the respiration slightly without affecting glycolysis. A more concentrated solution (*M*/30) in the presence of glucose inhibits respiration 35%. In both cases the R.Q. is considerably decreased. In the absence of glucose, *M*/30 pyrophosphate has practically no effect on respiration and glycolysis. It also has no effect on lactate metabolism. Elliott *et al.* [1935] and Elliott & Greig [1937] showed an extremely low succinoxidase content in Walker 256 tumour so that either respiration does not go through succinate or succinoxidase is likely to be the limiting factor. The definite effects of pyrophosphate, particularly on the R.Q., show that it does penetrate cells to a certain extent. If we can assume that it penetrates considerably we must expect succinoxidase inhibition; the absence of more marked effects on respiration then means that succinate is not an essential intermediate of respiration in this tissue.

With testis, which is also low in succinoxidase activity, pyrophosphate, in a concentration of *M*/30, inhibits O_2 uptake to some extent both in the presence and absence of glucose. Oxidation of lactate is inhibited by 27% by pyrophosphate indicating that removal of this acid in testis is probably to some extent dependent on the succinate system.

Pyrophosphate has little effect on the normal respiration of liver slices, but in the presence of glucose respiration is inhibited by 31% and acid formation is increased. With added lactate O_2 uptake is inhibited by about 24% and acid removal is practically completely inhibited. This indicates that the metabolism of liver, especially with added lactate, is, like that of testis, at least in part dependent on the succinoxidase system.

Pyrophosphate has no effect on the respiration of brain slices in the presence of glucose or on its metabolism of lactate. The fact that our experimental procedure and the duration of our experiment were quite different from those of Peters may explain the absence in our experiment of the stimulating effect of pyrophosphate on lactate metabolism. Since pyrophosphate does not inhibit

lactate oxidation in brain slices, this would seem to indicate that the main path of oxidation is not through succinate. However, the absence of any effect whatever might indicate that pyrophosphate does not penetrate the cells of sliced brain.

With kidney the effects are different from those found with the above tissues. In the absence of glucose pyrophosphate causes a slight inhibition in O_2 uptake and some accumulation of acid; in the presence of glucose there is increased respiration and increased glycolysis. These results may indicate that there is an alternative path of oxidation to that proceeding by way of succinate, or it may be that succinoxidase is not completely inhibited and perhaps other systems such as the malic and lactic systems are accelerated so that the net result is increased oxidation in the presence of glucose.

That the effect of pyrophosphate on glucose oxidation is not a salt effect was shown by adding NaCl approximately isotonic with the pyrophosphate used. This had no effect on respiration but it caused a small increase in acid formation.

With added succinate the effect of pyrophosphate is similar to that of malonate [Elliott & Greig, 1937]. The removal of acid groups is inhibited 60% and O_2 uptake is inhibited about 12%, but respiration is still greater than that in the absence of substrate or inhibitor, indicating that some succinate is still being oxidized. This is to be expected in view of the extremely high succinoxidase content of kidney [see Elliott and Greig, 1937]. In the presence of pyruvate, pyrophosphate inhibits removal of acid groups about 60%, i.e. about the same as for succinate, but the O_2 uptake remains the same as without inhibitor. Pyrophosphate decreases acid disappearance in the presence of malate by about 78% and respiration is decreased to about the same extent as with added succinate, although it is still higher than in the absence of substrate. These results are similar to those of Elliott and Greig with malonate and indicate that the succinoxidase system is concerned with the complete oxidation of malate to CO_2 and H_2O. With added lactate, pyrophosphate has no effect on O_2 uptake but decreases acid removal by 65% which also indicates the participation of the succinoxidase system in the complete oxidation of this substrate.

Ox retina differs from the other tissues studied in its reaction to pyrophosphate. Like testis it has a low succinoxidase content but added pyrophosphate, instead of inhibiting respiration, causes a large increase in O_2 uptake and a small increase in glycolysis in the presence of glucose. Without added substrate there is usually some increase in respiration. In all cases pyrophosphate raises the R.Q. With lactate as substrate pyrophosphate also causes a considerable increase in O_2 uptake and a small increase in acid disappearance.

It will be shown in a forthcoming paper that the rate of succinate oxidation by ox retina is not great enough for it to be a link in the chain of reactions for the oxidation of lactate. That pyrophosphate does not inhibit but indeed accelerates lactate and acid removal is further evidence that this reaction does not go through succinate.

From these results one might assume that in liver, testis and kidney succinate is an intermediate in carbohydrate metabolism, and pyrophosphate, in inhibiting succinoxidase, reduces respiration. Perhaps in kidney there is an alternative path for glucose oxidation. However, in brain and retina the succinate-fumarate system does not seem to play a necessary role. In the case of these tissues inhibition of the succinoxidase system does not inhibit respiration or acid disappearance. Pyrophosphate seems to act catalytically in retina in the oxidation of glucose or lactate, perhaps by formation of cocarboxylase or other coenzyme or by formation of some phosphorylated intermediate.

SUMMARY

Using the Dixon-Keilin manometric technique, it was found that pyrophosphate exerts various effects on the metabolism of tissue slices depending both on the tissue and the substrate employed.

1. It has no effect on the metabolism of brain slices.

2. The respiration of testis is inhibited in the presence or absence of added substrate.

3. Pyrophosphate has no effect on the normal respiration of tumour and, in low concentrations, has no effect on glucose oxidation, while in higher concentrations both glucose and lactate oxidations are inhibited.

4. It has little effect on the normal respiration of liver but inhibits respiration in the presence of glucose and lactate.

5. Pyrophosphate enhances the rate of glucose metabolism of kidney, has no effect on normal respiration and inhibits the complete oxidation of succinate, malate, pyruvate and lactate.

6. It has practically no effect on the normal respiration of retina but it considerably increases the rate of oxidation of glucose and lactate.

The authors wish to express their appreciation to Dr Ellice McDonald, Director, for his support and interest throughout the work, and to Dr K. A. C. Elliott for much helpful advice and criticism.

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XVII. AN ULTRAMICRO-KJELDAHL TECHNIQUE

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(*Received 26 November 1938*)

The problem of making Kjeldahl determinations of total nitrogen in amounts of the order of 1 μg. is not an easy one, and has not yet been satisfactorily solved. The present paper records a contribution to this end which has proved extremely useful in work described elsewhere on the metabolism of isolated regions of the amphibian gastrula [Needham *et al.* 1939; Boell & Needham, 1939; Boell *et al.* 1939]. As an indication of the delicacy of technique required, it may be recalled that the Pregl methods need amounts of from 100 to 300 μg. N [Pregl, 1937]. The amounts of total N which were to be estimated in the isolates from gastrulae varied from 1 to 20 μg.

Three previous papers have been devoted to this subject. Kirk [1934] worked out a method for Kjeldahl determinations on amounts of protein-N ranging from 3 to 15 μg. His method involved a rather undesirable transfer from the incineration vessel to a complicated steam distillation apparatus. He titrated some 300 μl. of distillate with 0·05 *N* NaOH. Levy [1936] abandoned transference and steam distillation; after dilution and neutralization of the digest, he estimated the nitrogen colorimetrically with the aid of Nessler solution and a Pulfrich photometer. Colorimetric estimations permitted him to measure amounts down to 0·4 μg., but most investigators would prefer to use a titration method, if possible. Lastly, Bentley & Kirk [1936] also abandoned steam distillation and had recourse to the diffusion principle, originally perfected by Conway & Byrne [1933] for ammonia determinations. They too, however, transferred the distillate from incineration vessel to diffusion vessel.

The essence of the problem may be said to lie in combining in one technique the Kjeldahl digestion, which logically demands a long narrow tube, and the Conway diffusion, which logically demands a maximal liquid-air interface, and hence a shallow vessel. At the same time it is desirable to neutralize the digest in a closed system so that none of the NH_3 first coming off shall escape.

The principle of our method lies in turning the Kjeldahl digestion vessel on its side after neutralization, and diffusing off the NH_3 from the resulting relatively large liquid-air interface into a hanging film of acid in a tube fitted into the mouth of the Kjeldahl vessel.

The vessel used by us[1] is shown in Fig. 1 (*a*). It consists of a digestion vessel with two bulbs blown in it (pyrex glass), some 7·5 cm. long. At the top there is an opening ground to receive a small tube of about the same dimensions as those used by Linderstrøm-Lang & Holter [1933] in their NH_3 estimations. Like their tubes also, it is coated on the inside with high melting-point paraffin wax. We refer to it as the receptor tube.

The digestion of the tissue proceeds as follows. The source of nitrogen is placed or pipetted into the bottom of the Kjeldahl vessel and 50–60 μl. of the digestion mixture added. The digestion mixture used by us was made up by

[1] Vessels of this kind can be obtained from Messrs C. Dixon, London, W.C.

adding 3 g. pure $CuSO_4$, 1 g. pure K_2SO_4 and 0·1 g. SeO_2 to 300 ml. pure conc. H_2SO_4. The tubes are then placed in an air oven at about 120° for *ca.* 3 hr. to drive off traces of water. At the end of that time a good deal of charring will be visible, and the digestion proper is carried out over pin flames constructed from old hypodermic needles (Fig. 1 (*b*)). Two glass beads are usually added, and we employ, as suggested by Levy, a mobile flame which the operator uses to heat the upper bulb when necessary. With the amounts of nitrogen used by us, digestion is complete in 3–5 min. If the tubes have been well heated in the air oven beforehand, and if they are constantly agitated during the heating by the use of the stem of the mobile flame, there is little danger of loss by sputtering, although as always where incinerations are concerned, care and practice cannot be dispensed with. When the liquid has become straw-coloured, the heating is continued for ½–1 hr. over a pin flame about 2 mm. high.

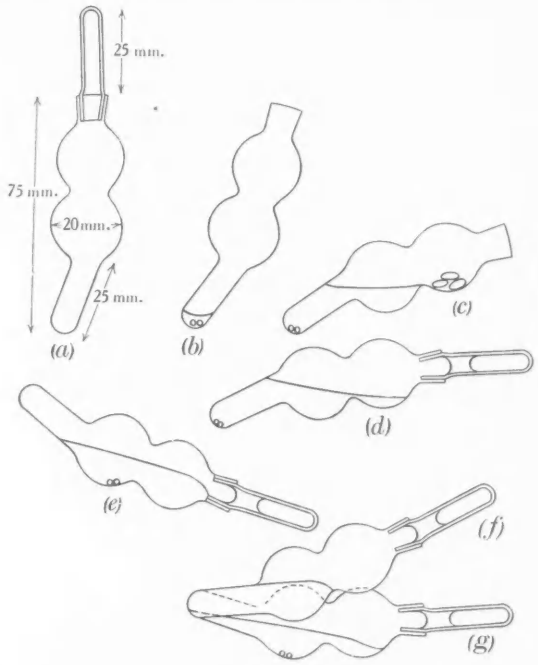

Fig. 1

The vessels are now removed from their holders and the digest diluted by the addition of 0·7 ml. NH_3-free distilled water. This is done with a pipette ending at an angle of 45° so as to facilitate the access of the water to all the interior surface of the vessel. The vessel is now tilted as shown in Fig. 1 (*c*) and three pellets of NaOH (corresponding to 300 mg. in all and giving 22 % NaOH) are placed in the upper bulb.

Meanwhile the receptor tubes have been prepared. 70 μl. of distilled water are placed as a hanging film at the top of the waxed interior and 7 μl. of 0·294 *N* HCl

delivered into the film from a constriction pipette [*vide* Linderstrøm-Lang & Holter, 1931]. The acid contains bromocresol purple in double the strength used in the Linderstrøm-Lang NH_3 method. It is important to put enough water in the receptor tube, as owing to the osmotic pressure difference, the film loses water as it gains in ammonia, eventually falling to perhaps 30–40 μl. The receptor tube is well sealed into the Kjeldahl vessel with a grease composed of one part high melting-point paraffin wax and two parts white vaseline. The NaOH and the diluted digest are carefully mixed, care being taken to see that the heat evolved does not blow out the receptor tube, see Fig. 1 (*d*). The whole is then cooled in water.

Now comes the most novel and delicate part of the technique. If traces of strong acid are left between the alkaline digest and the film of acid in the receptor tube, gross errors will result, for the ammonia will never reach its proper destination. The vessel is therefore held as in Fig. 1 (*e*) and rotated so that the alkaline digest runs right round the opening of the receptor tube. The wax coating of the interior of the receptor tube, and the rim of wax grease around the ground joint alike prevent the entry of the slightest trace of strongly alkaline digest into the receptor tube. It is essential to cool the vessel well before carrying out this part of the technique, for otherwise the wax grease and the wax coating may melt, with unfortunate results.

It is now simply a question of time before the acid in the receptor tube is ready for titration. We place the vessels, however, in a horizontal position in a rocker at 37° overnight and titrate the following day. The rocker was easily made from an electric gramophone motor connected to an eccentric so as to give the motion seen in Fig. 1 (*f*, *g*). At each slow rise and fall the alkaline digest spills into the upper bulb and then falls back again into the bottom of the vessel.

At the end of the period of diffusion desired, the receptor tubes are removed, the ends carefully wiped, and the films titrated against 0·102 *N* NaOH from a Rehberg-Heatley micro-burette of 50 μl. capacity [*v.* Heatley, 1939]. Agitation of the film is accomplished magnetically as described by Linderstrøm-Lang & Holter [1931].

Using a solution of NH_4Cl we first determined the time taken under our conditions for 100 % of the NH_3 to diffuse at 37° from the alkaline digest into the acid film in the receptor tube. As is shown by Fig. 2, 95·2 % recovery was reached

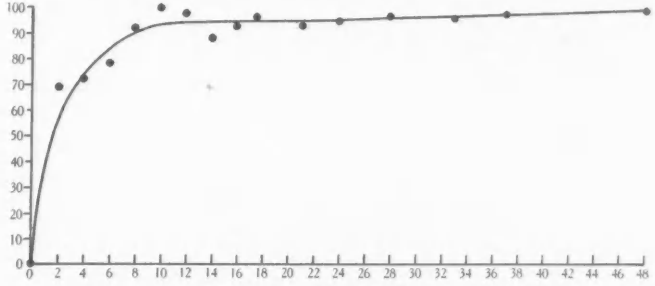

Fig. 2. Average recovery after 10 hours' distillation = 95·2 %.

in 10 hr. so that our usual practice of letting the rocking proceed for 18 hr. appears to be well on the safe side. The fact that the time required is a good deal longer than that in the NH_3 methods of Conway & Byrne [1933] and Conway

[1935] must certainly be due to the fact that the Conway vessel has a large donator surface and a large receptor surface; under our conditions only a large donator surface is possible.

Table I shows the percentage recoveries from solutions of casein (Hammarsten).

Table I

Exp.	Casein (μg.)	Recovery (μg.)	Percentage
II	0·58	0·525	90
	1·17	1·11	95
III	1·13	1·19	105
	1·13	1·07	95
	1·14	1·12	98
I	2·06	2·36	115
	3·09	3·09	100
	3·09	3·11	101
	4·12	4·11	100
	6·57	5·83	89
	6·57	6·00	91
	6·57	6·56	100
	6·57	5·84	89
	6·57	5·85	89
	6·57	6·52	99
	6·57	6·25	95
	6·57	6·38	97
	6·57	6·62	101
	6·57	6·40	97
		Av.	97·5

We see no reason why this method should not be applied to the estimation of quantities of total N much less than 1 μg., but we believe that many further precautions would be necessary, especially as regards the purity of the reagents and the contamination of the atmosphere, as by smoking etc.

SUMMARY

An ultramicro-Kjeldahl technique, suitable for amounts of total N from 1 to 20 μg. is described. It includes a new type of Kjeldahl vessel, which, when turned on its side, acts as a Conway dish with a relatively large donator surface. The NH_3 diffuses into an acid film hanging in a modified Linderstrøm-Lang wax-coated receptor tube, which blocks up the mouth of the Kjeldahl vessel. Tests of the method, with a working description, are given in the text.

Acknowledgement is made to the Government Grant Committee of the Royal Society for a grant which partially defrayed the cost of the work.

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XVIII. THE VITAMIN C REQUIREMENTS OF MAN

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(*Received 30 December 1938*)

THE subject of this communication is a continuation of an inquiry into the relation of the intake of ascorbic acid to the urinary output and to the storage of the vitamin in the body.

The first paper of this series [Johnson & Zilva, 1934] dealt with the excretion of ascorbic acid by the kidney in man. The second inquiry [Zilva, 1936] was concerned not only with the excretion but with the storage of vitamin C in the body of the guinea-pig. In this animal only could storage questions be satisfactorily studied.

These investigations revealed that there was no standard of normality as regards the urinary excretion of ascorbic acid by man but that the output was conditioned by the amount stored in the body and by the quantity consumed in the diet. This conclusion was in agreement with the observations made by Van Eekelen *et al.* [1933] and by Hess & Benjamin [1934]. It was further found that when the store of the vitamin in the body was low, some time elapsed before a constant level of excretion indicating "saturation" was reached when the dose was small and that this time could be markedly shortened when large doses were offered. Quantities of ascorbic acid apparently larger than those required by the organism were not accounted for in the urine when the subject was "saturated" and the amount of vitamin thus not utilized was greater when larger doses were taken. On discontinuing the intake of ascorbic acid "saturation" persisted for a short time although the output in the urine almost disappeared. On the other hand, subjects only partially "saturated" could excrete considerable quantities of ascorbic acid when large doses were taken. The experiments on guinea-pigs disclosed in addition the fact that ascorbic acid first appeared in the urine when the maximum concentration of the vitamin was reached in the tissues. No doubt this also holds in the case of man.

These data brought into relief two interesting points. First, in order to determine the daily requirements of vitamin C by balancing the intake and the urinary excretion of ascorbic acid, the suitable dose should be established by trial and error. The dose should also be small, in order to reduce to a minimum the amount of ascorbic acid which, although not accounted for in the urine, is not actually involved in the metabolic function of the vitamin. Secondly, the minimum protective dose of ascorbic acid cannot be easily defined. It has been established that it is possible to exist for months on diets containing quantities of vitamin C sufficient to maintain good health and to prevent scurvy in spite of the fact that the store of ascorbic acid is very low. In guinea-pigs the daily dose required to bring about "saturation" is about 20 times that necessary to protect them from macroscopic scurvy. As a disparity between the two respective doses undoubtedly exists also in the case of man it becomes a debatable point as to what constitutes the optimum dose.

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The obvious extension of the work lay in the direction of ascertaining the minimum daily dose required to maintain a man "saturated" in respect to vitamin C, although, as will be pointed out later, such a dose is in all probability well above the optimum dose. With this end in view a number of experiments on the metabolism of vitamin C were carried out by the writers. In addition the ascorbic acid content of the blood was determined at various stages of these experiments in order to throw further light on the problem. The results obtained in the course of this investigation form the subject of this communication.

Methods

As was already mentioned, an individual may be considered as "saturated" when the amount of ascorbic acid voided in the urine after a continued consumption of a certain dose becomes more or less constant. The first appearance of the acid is not always indicative of "saturation". The level of ascorbic acid excretion reached at "saturation" for a certain dose will, however, vary in certain instances according to the previous intake of ascorbic acid. If this has been high the level of excretion at equilibrium will be higher, at least for a very considerable time, than it would be if the intake had been lower. If on reaching "saturation" the intake of ascorbic acid is stopped, excretion persists for a few days whilst the output gradually diminishes. Therefore, on descending from a higher to a lower dose there is a certain amount of overlapping in the urinary output of the vitamin until, after a gradual diminution, a more or less constant level of excretion is attained. On the other hand, on ascending from a lower to a higher dose the output increases until equilibrium is reached. In order to obtain a more accurate conception, the level of excretion for each dose was determined by employing the dose both by ascending from a condition of "unsaturation" and by descending from a condition of "saturation" with as large a dose as 600 mg. per day. The doses employed were 30, 50 and 100 mg. since it was anticipated that the minimum daily dose capable of bringing about "saturation" lay within this range. The preliminary part of this investigation was carried out on S. S. Z. but for reasons of health it was decided to carry out the final experiments on A. E. K. (weight 184 lb.).

The indophenol titration procedure was similar to that employed by Johnson & Zilva [1934] who have previously shown by biological tests that under normal conditions the indophenol-reducing capacity could be ascribed mainly to ascorbic acid. There is, however, indirect evidence that not all of the reduction in the urine is necessarily due to ascorbic acid. Thus, in perusing the literature, one finds records of cases in which the urine from scorbutic patients reduced indophenol to some extent and this was assumed by some of the workers to be due to ascorbic acid. This assumption seems to be highly improbable, especially as it was shown by Johnson [1936] and by Kellie & Zilva [1938] that a reducing substance (or substances) which is not ascorbic acid persists in the lens of the eye and in the leucocytes of the guinea-pig after the remaining tissues of the animals have been exhausted of vitamin C by dietetic means. In the present investigation it was also found that when S. S. Z. and A. E. K. were "unsaturated" their urines reduced the indicator with comparatively little variation to the extent of 1 ml. $N/1000$ indophenol to 10 ml. of urine. This reduction value was assumed by us not to be due to ascorbic acid and was accordingly deducted from all the results.

The determination of ascorbic acid in the blood was carried out as follows. 10 ml. of blood were taken from a vein and collected directly into a centrifuge

tube containing 3 drops of saturated potassium citrate solution. The plasma which was obtained by centrifuging was then extracted with an equal volume of 10 % trichloroacetic acid and centrifuged. The extract was adjusted to *p*H 2–3 and titrated directly with 1·14 *N*/1000 indophenol. In one or two samples a certain amount of haemolysis took place during the collection of the blood. In these cases the trichloroacetic acid extract was adjusted to *p*H 4·5 and reduced with H_2S before being titrated. We should like to take this opportunity of thanking Dr C. R. Amies for collecting our samples of blood.

Results

Exp. 1. In this experiment A. E. K., existing on a diet low in vitamin C, took a daily dose of 600 mg. of ascorbic acid until equilibrium was established in the urinary output. The dose was then reduced to 100 mg. It will be seen from Table I that during the first 3 days (10–12 September) on a daily dose of 100 mg. there was an overlapping in the output due to the extra excretion of the excess of ascorbic acid accumulated during the time when the subject was brought up to "saturation" by the daily consumption of 600 mg. A new level of excretion, namely 50–60 mg. per day, was then reached which persisted for 23 days. During part of this time, namely between 17 September and 4 October, the output was not measured, although the daily dose of 100 mg. was taken as usual. The measurement of the urinary excretion was resumed on 4 and 5 October, when it was found that the amount of ascorbic acid was still of the same order as that observed previously, indicating the probability that this level had been maintained all the time.

Exp. 2. In this experiment, which followed Exp. 1, the daily dose of ascorbic acid was reduced to 50 mg. and the output was studied for 17 days. The data (Table II) show that in this case the urinary excretion of ascorbic acid diminished very gradually to about 30 % of the intake, when it reached equilibrium after a fortnight. It will be seen later that a similar figure was obtained when this experiment was repeated a few months afterwards (Exp. 8).

Table I. *Ascorbic acid excretion in urine. Daily dose of 100 mg. following "saturation" on 600 mg. per day*

Date (1937)	Intake of ascorbic acid mg.	Vol. of urine ml.	Output of ascorbic acid mg.	% ascorbic acid excreted by kidney
1 Sept.	600	1985	23·9	4·0
2	600	1678	249·5	41·5
3	600	1474	374·2	62·5
4	600		Not determined	
5	600		Not determined	
6	600	1067	462·6	76·8
7	600	1513	502·9	83·8
8	600	1293	526·0	87·6
9	600	1691	501·3	83·4
10	100	1942	110·6	110·6
11	100	1177	66·5	66·5
12	100	1915	75·1	75·1
13	100	1963	30·7	30·7
14	100	1450	48·8	48·8
15	100	1601	68·5	68·5
16	100	2137	44·3	44·3
17	100	1327	58·1	58·1
4 Oct.	100	1496	56·0	56·0
5	100	1401	58·4	58·4

Table II. *Ascorbic acid excretion in urine. Daily dose of 50 mg. following "saturation" on 100 mg. per day*

Date (1937)	Vol. of urine ml.	Output of ascorbic acid mg.	% ascorbic acid excreted by kidney
6 Oct.	1309	76·3	152·6
7	1343	55·1	110·2
8	1095	36·0	72·0
9		Not determined	
10		Not determined	
11	1470	32·9	65·8
12	1147	31·1	62·2
13	1784	30·6	61·2
14	1041	26·9	53·8
15	1380	25·3	50·6
16		Not determined	
17		Not determined	
18	1529	26·4	52·8
19	1415	21·9	43·8
20	1435	16·7	33·4
21	1331	15·0	30·0
22	1547	15·1	30·2

Table III. *Ascorbic acid excretion in urine. Daily dose of 50 mg. following a period of "unsaturation"*

Date (1937)	Vol. of urine ml.	Output of ascorbic acid mg.	% ascorbic acid excreted by kidney
29 Nov.	1415	2·5	5·0
30	1330	1·6	3·2
1 Dec.	1580	2·4	4·8
2	1410	2·5	5·0
3	1410	2·4	4·8
4		Not determined	
5		Not determined	
6	1844	2·1	4·2
7	1434	5·5	11·0
8	1555	4·2	8·4
9	1745	4·2	8·4
10	2029	5·1	10·2
11	2014	5·5	11·0
12		Not determined	
13	1601	5·2	10·4
14	1582	4·2	8·4
15	1910	3·4	6·8

Table IV. *Ascorbic acid excretion in urine. Daily dose of 30 mg. following "saturation" on 50 mg. per day*

Date (1937)	Intake of ascorbic acid mg.	Vol. of urine ml.	Output of ascorbic acid mg.	% ascorbic acid excreted by kidney
16 Dec.	30	1604	2·2	7·3
17	30	1850	1·6	5·3
18	30		Not determined	
19	30		Not determined	
20	30	1678	1·1	3·7
21	30	1740	0·3	1·0
22	30	1501	0·4	1·3
23	600	1349	145·7	24·0
24	600	2430	331·4	55·0
25	600	1570	374·9	62·5

Exp. 3. The subject then placed himself on a diet deficient in vitamin C from 23 October to 28 November in order to attain a condition of "unsaturation". On 29 November (Table IV) he resumed his daily dose of 50 mg. of ascorbic acid. During the first 8 days only about 5 % of the intake was excreted by the kidney, but after that time the output in the urine rose to about 10 % and was maintained at this level for 9 days. The daily quantity of ascorbic acid thus excreted—as will also be seen from Exp. 5—was considerably less than that observed in Exps. 2 and 8 when the same dose of the vitamin was taken after the subject had been previously "saturated" on higher doses, namely 600 mg. followed by 100 mg.

Exp. 4. This experiment, in which the daily dose of ascorbic acid was reduced to 30 mg., followed immediately on Exp. 3. After a week on this dose it was found (Table IV) that the output of the vitamin in the urine fell almost to zero. The subject at this time would appear to have been not far removed from "saturation", since of the 3 doses of 600 mg. taken on consecutive days 24 % was excreted on the first day, 55 % on the second and 62·5 % on the third.

Exp. 5. This experiment (Table V) was a repetition of Exp. 3 in so far that the subject was not "saturated". Whilst, however, in Exp. 3 he existed on a diet deficient in vitamin C for over a month, in this case his condition was not so far removed from "saturation", since he deprived himself of vitamin C for 8 days only after having been previously fully "saturated" (cf. Exp. 4). The daily output of ascorbic acid in the urine was, nevertheless, of the same order as in Exp. 3. The excretion of 82·2 % of a dose of 600 mg. of ascorbic acid after three daily consecutive doses afforded ample proof that the subject was "saturated".

Exp. 6. Exp. 2 on the one hand, and Exps. 3 and 5 on the other (daily dose 50 mg.), have shown that the level in the urinary excretion of ascorbic acid at equilibrium was dependent upon whether the patient was "saturated" or not at the beginning of the experiment. In Exp. 6 an attempt was made to ascertain whether this held with a larger dose, namely 100 mg. The subject began taking this dose after having subsisted on a vitamin C-deficient diet for 6 days. The results in Table VI show that also on a dose of 100 mg., when the patient was only partially "saturated", the output of ascorbic acid in the urine at equilibrium was less than that when he was fully "saturated" (cf. Tables I and VII).

Table V. *Ascorbic acid excretion in urine. Daily dose of 50 mg. following a period of "unsaturation"*

Date (1938)	Intake of ascorbic acid mg.	Vol. of urine ml.	Output of ascorbic acid mg.	% ascorbic acid excreted by kidney
4 Jan.	50		Not determined	
5	50	1308	3·4	6·8
6	50	1379	4·4	8·8
7	50	1217	4·2	8·4
8	50		Not determined	
9	50		Not determined	
10	50	1490	3·3	6·6
11	50	1784	2·7	5·4
12	50	1652	4·4	8·8
13	50	1602	4·6	9·2
14	50	1713	5·5	11·0
15	50		Not determined	
16	50		Not determined	
17	50	2041	3·3	6·6
18	600	1964	140·0	23·3
19	600	1683	374·0	62·3
20	600	1752	494·9	82·2

Table VI. *Ascorbic acid excretion in urine. Daily dose of 100 mg. following a period of "unsaturation"*

Date (1938)	Intake of ascorbic acid mg.	Vol. of urine ml.	Output of ascorbic acid mg.	% ascorbic acid excreted by kidney
27 Jan.	100	Not measured	0	0
28	100	Not measured	0	0
29	100		Not determined	
30	100		Not determined	
31	100	1605	21·4	21·4
1 Feb.	100	1825	34·8	34·8
2	100		Not determined	
3	100	2180	39·2	39·2
4	100	1790	43·7	43·7
5	100		Not determined	
6	100		Not determined	
7	100	1805	48·3	48·3
8	100	2065	52·8	52·8
9	100	1814	38·9	38·9
10	100	2135	53·6	53·6
11	100	2107	44·2	44·2
12	100		Not determined	
13	100		Not determined	
14	100	2012	35·2	35·2
15	100	1975	49·7	49·7
16	100	1650	39·1	39·1
17	100	1950	30·0	30·0
18	100	1984	24·6	24·6
19	100	2100	39·3	39·3
20	100		Not determined	
21	100	1706	41·1	41·1
22	100	1802	39·2	39·2
23	100	2585	23·7	23·7
24	600	2229	427·4	71·2
25	600	1810	448·7	74·6
26	600	1760	543·7	90·6

Table VII. *Ascorbic acid excretion in urine. Daily dose of 100 mg. following "saturation" on 600 mg. per day*

Date (1938)	Intake of ascorbic acid mg.	Vol. of urine ml.	Output of ascorbic acid mg.	% ascorbic acid excreted by kidney
27 Feb.	100	2090	137·8	137·8
28	100	1602	71·7	71·7
1 Mar.	100	1935	68·8	68·8
2	100	1735	72·4	72·4
3	100	1879	53·4	53·4
4	100	1669	49·4	49·4
5	100		Not determined	
6	100		Not determined	
7	100	1595	40·7	40·7
8	100	2415	50·8	50·8
9	100	2420	60·2	60·2
10	100	2017	43·4	43·4
11	100	1715	55·7	55·7
12	100		Not determined	
13	100		Not determined	
14	100	1180	37·8	37·8
15	100	1325	40·2	40·2
16	600	2442	409·1	68·0
17	600	1226	462·8	77·0
18	600	1620	481·2	80·1

Table VIII. *Ascorbic acid excretion in urine. Daily dose of 50 mg. following "saturation" on 600 mg. per day*

Date (1938)	Intake of ascorbic acid mg.	Vol. of urine ml.	Output of ascorbic acid mg.	% ascorbic acid excreted by kidney
19 Mar.	50		Not determined	
20	50		Not determined	
21	50	1300	31·1	62·2
22	50	2125	17·7	35·4
23	50	2005	18·6	37·2
24	50	1825	13·8	27·6
25	50	2195	10·7	21·4
26	50		Not determined	
27	50		Not determined	
28	50	1734	9·9	19·8
29	50	1845	13·0	26·0
30	50	2164	13·9	27·8
31	50	1945	10·1	20·2
1 Apr.	50	1987	11·3	22·6
2	50		Not determined	
3	50		Not determined	
4	50	1922	13·2	26·4
5	600	2015	224·1	37·3
6	600	1750	339·5	56·5
7	600	2287	513·8	85·4

Exp. 7. This experiment was a repetition of Exp. 1 and was carried out immediately after Exp. 6. The subject was then fully "saturated" (cf. Table VI). The urinary excretion at equilibrium was found to approximate (Table VII) to that observed in Exp. 1 (Table I) and not to that found in Exp. 6 when the subject was not "saturated".

Exp. 8. In this experiment, performed immediately after Exp. 7, the dose was reduced to 50 mg. It was therefore a repetition of Exp. 2. Although equilibrium in the urinary excretion of ascorbic acid was attained in this case (Table VIII) earlier than in Exp. 2, the output at equilibrium was of the same order in both experiments.

Tables IX–XI give the ascorbic acid contents of the blood of the two experimental subjects during the course of the investigation. These figures yield information particularly concerning two points. Without going into a detailed analysis of the literature on the vitamin C content of the blood, which is very extensive and often contradictory, it may be stated that some workers maintain that there is a renal threshold for ascorbic acid in man and that the ascorbic acid concentration of the blood is an index of the degree of "saturation" of the subject in respect to vitamin C. The data in Tables IX–XI throw some light on both these problems.

The fact that a subject is capable of excreting ascorbic acid in the urine before reaching "saturation" when the dose taken is large, suggests that the concentration of the vitamin in the blood must to some extent control its excretion by the kidney. The threshold values advanced by a number of workers vary from 0·9 mg. to 1·5 mg. per 100 ml. of blood. Even if the individual variation of the subject and the variation in the methods employed by these workers be taken into account, the difference in the results obtained by them would still seem to be too great. Our results may offer some explanation for this disparity. It is seen that when S. S. Z. (23 February 1937, Table IX), on reaching "saturation", discontinued his dose, the ascorbic acid content of his blood then fell from 1·63 mg. per 100 ml. of plasma to 0·45 mg. when the dose was 600 mg.

Table IX. *Relation of the ascorbic acid content of the blood of "saturated" subjects to their 24 hr. urinary output*

Subject and date (1937)	Time of collection	Ascorbic acid in plasma mg./100 ml.	Ascorbic acid in urine mg./24 hr.	Remarks
S. S. Z.				
23 Feb.	11.25 a.m.	1·63	525·1	"Unsaturated" level of ascorbic acid =0·35 mg./100 ml. plasma. 15–23 Feb. (incl.) 600 mg. ascorbic acid daily at 10 a.m.
24	11.35 a.m.	0·80	74·9	
25	12 noon	0·53	40·9	
26		Not determined	18·6	
27	11.35 a.m.	0·45	Not determined	
			mg. excreted from 10 a.m. to 5 p.m.	
8 Mar.	12.30 p.m.	0·73	16·6	27 Feb.–8 Mar. (incl.) 100 mg. ascorbic acid daily at 10 a.m.
9		Not determined	21·9	
10	11.15 a.m.	0·54	7·2	
A. E. K.			mg./24 hr.	
9 Sept.	11 a.m.	1·53	501·3	"Unsaturated" level of ascorbic acid =0·47 mg./100 ml. plasma. 1–9 Sept. (incl.) 600 mg. ascorbic acid daily at 11 a.m.
17	10 a.m.	1·28	58·1	10–17 Sept. (incl.) 100 mg. ascorbic acid daily at 10 a.m.
15 Oct.	10 a.m.	0·80	25·3	18 Sept.–3 Oct. (incl.) 2 oranges daily. 4–5 Oct. (incl.) 100 mg. ascorbic acid daily. 6–15 Oct. (incl.) 50 mg. ascorbic acid daily at 10 a.m.
21	10 a.m.	1·08*	15·0	16–22 Oct. (incl.) 50 mg. ascorbic acid daily at 10 a.m.
22	10 a.m.	1·06	15·1	
14 Dec.	10 a.m.	0·47*	4·2	23 Oct.–28 Nov. (incl.) vitamin C-deficient diet. 29 Nov.–15 Dec. (incl.) 50 mg. ascorbic acid daily at 10 a.m.
15	10 a.m.	0·62	3·4	
22	10 a.m.	0·67	0·4	16–22 Dec. (incl.) 30 mg. ascorbic acid daily at 10 a.m.

* Some haemolysis.

and from 0·73 to 0·54 mg. when the dose was 100 mg., i.e. to values considered by many workers as an indication of vitamin C deficiency. In spite of these low blood levels eventually reached, the subject was fully "saturated" and still excreted ascorbic acid in the urine. It would therefore appear that in this case the level of the so-called "renal threshold" was influenced by the ascorbic acid content of the tissues. A similar conclusion can be drawn from A. E. K.'s data (Table IX). That the excretion of ascorbic acid by the kidney is not entirely influenced by the blood ascorbic acid level is apparent when a comparison is made between the blood content and the quantities of ascorbic acid excreted by "saturated" and "unsaturated" subjects immediately before and after the samples of the blood were taken (Tables X and XI). These results suggest that in all probability there is a competition for the ascorbic acid of the blood by the absorptive tendency of the tissues and the excreting function of the kidney.

The results also show that blood levels determined at random, even if they be "fasting" values, may sometimes lead to wrong conclusions concerning the condition of "saturation" of the subject. The data given in Tables IX–XI

Table X. *Relation of the ascorbic acid content of the blood of "saturated" subjects to their urinary output*

Subject and date (1937)	Ascorbic acid intake: Time	Ascorbic acid intake: Dose mg.	Time of collection	Ascorbic acid: Plasma mg./100 ml.	Ascorbic acid: Urine mg./sample	Remarks
S. S. Z.						
23 Feb.	10 a.m.	600	11.25 a.m. 12.45 p.m. 2.30 p.m.	1·63 Not determined 1·45	25·9 65·3 121·6	"Unsaturated" level, 0·35 mg./100 ml. plasma. 15–22 Feb. (incl.) 600 mg. ascorbic acid daily. Subsequent urinary excretion: 2.30–5 p.m. 113·7 mg.; 5–10 p.m. 103·1 mg.; 10 p.m.–8 a.m. (24 Feb.) 73·8 mg.; 8–10 a.m. 21·7 mg.
A. E. K.						
9 Sept.	11 a.m.	600	11 a.m. 12 noon 2 p.m.	1·51 Not determined 2·89	No urine passed 6·1 189·5	"Unsaturated" level, 0·47 mg./100 ml. plasma. 1–8 Sept. (incl.) 600 mg. ascorbic acid daily. Subsequent urinary excretion: 2–4.30 p.m. 174·8 mg.; 4.30–6 p.m. 54·9 mg.; 6–9 p.m. 40·2 mg.; 9–11 p.m. 15·4 mg.; 11 p.m.–12.20 a.m. (10 Sept.) 6·4 mg.; 12.20–10 a.m. 14 mg.
17 Sept.	10 a.m.	100	10 a.m. 12 noon 1 p.m.	1·28 Not determined 1·71	0·2 2·1 No urine passed	10–16 Sept. (incl.) 100 mg. ascorbic acid daily. Subsequent urinary excretion: 12–2 p.m. 4·5 mg.; 2–4 p.m. 18·3 mg.; 4–6.30 p.m. 17·2 mg.; 6.30–9 p.m. 6·3 mg.; 9 p.m.–8 a.m. (18 Sept.) 8·2 mg.; 8–10 a.m. 1·5 mg.
22 Oct.	10 a.m.	50	10 a.m. 12 noon 1 p.m.	1·06 Not determined 1·29	0·7 1·0 No urine passed	18 Sept.–3 Oct. (incl.) 2 oranges daily; 4–5 Oct. (incl.) 100 mg. ascorbic acid daily; 6–21 Oct. (incl.) 50 mg. ascorbic acid daily. Subsequent urinary excretion: 12–2 p.m. 2·1 mg.; 2–4 p.m. 2·8 mg.; 4–6 p.m. 1·0 mg.; 6–10 p.m. 2·5 mg.; 10 p.m.–8 a.m. (23 Oct.) 5·2 mg.; 8–10 a.m. 0·5 mg.
15 Dec.	10 a.m.	50	10 a.m. 12 noon 1 p.m.	0·62 Not determined 0·80	0 0 No urine passed	23 Oct.–28 Nov. vitamin C-deficient diet. 29 Nov.–14 Dec. (incl.) 50 mg. ascorbic acid daily. Subsequent urinary excretion: 12–2 p.m. 1·2 mg.; 2–4 p.m. 0·4 mg.; 4–6 p.m. 0·3 mg.; 6–10 p.m. 0 mg.; 10 p.m.–8 a.m. (16 Dec.) 1·4 mg.; 8–10 a.m. 0·1 mg.
22 Dec.	10 a.m.	30	10 a.m. 12 noon 1 p.m.	0·67 Not determined 0·72	0 0 0	16–21 Dec. (incl.) 30 mg. ascorbic acid daily. Subsequent urinary excretion: 1–10 p.m. 0 mg.; 10 p.m.–8 a.m. (23 Dec.) 0·4 mg.; 8–10 a.m. 0 mg.

Table XI. *Relation of the ascorbic acid content of the blood of an "unsaturated" subject (A. E. K.) to his urinary output*

Date (1937)	Time of collection	Ascorbic acid: Plasma mg./100 ml.	Ascorbic acid: Urine mg./sample
26 Jan.	10 a.m.	Not determined	0
	1 p.m.	,,	0
	2.15 p.m.	0·57	No urine passed
	4.45 p.m.	Not determined	0
	6 p.m.	,,	0
27	*600 mg. ascorbic acid taken at 10 a.m.*		
	10.30 a.m.	0·92	0
	11 a.m.	Not determined	0
	11.45 a.m.	,,	0
	12.25 p.m.	,,	0
	12.45 p.m.	1·27	No urine passed
	1.15 p.m.	Not determined	0
	2.30 p.m.	,,	0
	3.15 p.m.	,,	0
	4 p.m.	1·12	No urine passed
	7 p.m.	Not determined	2·1
	10.30 p.m.	,,	0
28	8.30 a.m.	,,	3·2
	10 a.m.	1·0	No urine passed
	10.45 a.m.	Not determined	1·2
	1 p.m.	,,	0
	3.20 p.m.	,,	0·3
	5 p.m.	0·83	No urine passed
	5.50 p.m.	Not determined	1·8
	8.30 p.m.	,,	0·4
29	10 a.m.	,,	0·5
	12.30 p.m.	0·77	0

Date (1937)	Time of collection	Ascorbic acid: Plasma mg./100 ml.	Ascorbic acid: Urine mg./sample
26 Aug.	10 a.m.–6 p.m.	Not determined	0
27	10 a.m.–6 p.m.	,,	0·3
28	10 a.m.–12 noon	,,	0·3
29	10 a.m.–6 p.m.	,,	0·7
30	*100 mg. ascorbic acid taken at 11 a.m.*		
	10 a.m.	Not determined	0
	11 a.m.	0·52	No urine passed
	12 noon	0·67	0
	2 p.m.	0·72	0
	4 p.m.	Not determined	0
	6 p.m.	,,	0
	10 p.m.	,,	0
31 Aug.	8 a.m.	,,	0
	10 a.m.	,,	0
	11 a.m.	0·50	0
	12 noon	Not determined	0
	2 p.m.	,,	0
	4 p.m.	,,	0
	6.15 p.m.	,,	0
	10 p.m.	,,	0
1 Sept.	8 a.m.	,,	1·1
	600 mg. ascorbic acid taken at 10.30 a.m.		
	10.30 a.m.	0·47	0
	12 noon	Not determined	0·4
	1.30 p.m.	1·69	No urine passed
	2 p.m.	Not determined	7·5
	4 p.m.	,,	11·6
	6 p.m.	,,	2·9
	8 p.m.	,,	0
	10.30 p.m.	,,	0·8
2 Sept.	2.45 a.m.	,,	0
	10 a.m.	,,	1·5
	10.30 a.m.	1·10	No urine passed

afford further information about the variation of the ascorbic acid content of the blood with the magnitude of the dose and the time elapsing after taking the dose. They show, as has already been observed by numerous workers, that the vitamin C content of the blood rises after oral administration of the vitamin, reaching a maximum 2–4 hr. later. The maximum value thus attained is influenced by the magnitude of the dose and by the "fasting" value; it also varies with the individual.

Conclusions

The experiments bring out very plainly the difference between the level of excretion of ascorbic acid at equilibrium when a certain dose was taken after ascending from a condition of "unsaturation" or from a condition of "saturation" reached on a smaller dose and that recorded with the same dose taken after having previously attained "saturation" with a larger dose. This disparity in the level of output no doubt explains, at least in part, the variation in the amount of the apparent retention observed by different workers on the same dose of ascorbic acid.

As was to be expected, these levels of excretion decreased with the diminution in the intake. On a daily dose of 30 mg. the appearance of ascorbic acid in the urine almost ceased. On the other hand, on a dose of 50 mg. 20–30 % of the intake was excreted by the kidney at equilibrium when descending from a higher dose and 8–10 % when ascending from "unsaturation". From this it may be assumed that the minimum dose necessary to bring about "saturation" lies in this case between 30 and 50 mg. per day, probably nearer the former figure. Schultzer [1937] was able to "saturate" a scorbutic patient (weight 63 kg.) in 4 weeks with a daily dose of 40 mg. of ascorbic acid administered intravenously. In all probability ascorbic acid is utilized more efficiently when introduced parenterally than when it is given orally.

Although in practice it is advisable to aim at a dose of this order it is by no means to be assumed that this is the minimum daily dose necessary to prevent the onset of scurvy or even to maintain good health. It would perhaps be appropriate at this stage to review a few facts established in this connexion concerning the vitamin C requirements of the guinea-pig. Zilva, by determining the ascorbic acid content of the tissues of these animals, found that the condition of "saturation", i.e. when the tissues carry the maximum load of the vitamin, could only be maintained by administering *per os* a daily dose of 20 mg. or more. When the dose was reduced to about 8 mg. the tissues lost after 15 days the major part of their ascorbic acid, only traces being present; yet this dose is 4 times the daily minimum dose necessary to protect a guinea-pig fully from macroscopic and microscopic scorbutic lesions. On the assumption that the same proportions hold in the case of man one would expect that about a half of the "saturation" dose, i.e. 15 mg., would be sufficient not only to prevent scurvy but to maintain an individual in good health. This dose may at first sight appear to be rather low. On reflexion, however, it will be found that in certain circumstances the daily food of apparently healthy individuals does not contain over long periods much more vitamin C than this. The above figure of 15 mg. cannot therefore be far removed from the actual theoretical requirement of man. The view advanced by one of us [Zilva, 1936; 1937] may here be stressed once more, that no undue clinical significance need be attached to the importance of maintaining the condition of "saturation". As already mentioned, however, in order to ensure a margin of safety, it may be advisable to insist in practice on the minimum "saturation" dose, which in this investigation was found to be

30–40 mg. per day, as the requisite vitamin C intake for healthy adults. In the case of pregnant women and of nursing mothers this would of course be considerably higher.

Summary

The level of urinary excretion of ascorbic acid on a certain dose was found to be lower after ascending from a condition of "unsaturation", or from a condition of "saturation" reached on a lower dose, than when the same dose was taken after having previously attained "saturation" with a higher dose.

The equilibrium in the excretion of a subject weighing 184 lb. was established on the following daily doses, 30 mg., 50 mg. and 100 mg.

On a daily intake of 30 mg. the appearance of ascorbic acid in the urine almost ceased. It was assumed that the minimum "saturation" dose lay in the neighbourhood of this figure.

Although in practice it would be advisable to recommend the "saturation" dose as the minimum vitamin C daily intake for normal individuals it is argued that about half of this dose is sufficient not only to prevent scurvy but to maintain them in good health.

The ascorbic acid content of the blood was determined in two individuals during "saturation" and "unsaturation" at different intervals after the administration of the vitamin in various doses. The comparison of these figures with those of the urinary excretion suggests that there is no constant renal threshold for ascorbic acid, but that there is a competition for the ascorbic acid of the blood by the absorptive capacity of the tissues and the excreting function of the kidney.

Blood levels determined at random do not indicate the degree of "saturation" of a subject.

One of us (A. E. K.) is indebted to the Medical Research Council for a whole time grant. We should also like to express our thanks to Messrs Roche Products Ltd. for a gift of ascorbic acid.

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XIX. THE ACTION OF SOME *ENDO*SUCCINIC ACIDS DERIVED FROM POLYCYCLIC HYDROCARBONS ON THE RED BLOOD CORPUSCLES OF THE MOUSE

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(*Received 17 December 1938*)

Strong [1936] and Strong & Francis [1937] have described a fall in the haemoglobin content of the blood of female mice of strains which are liable to the development of spontaneous mammary carcinoma. The experiments of Parsons and her collaborators have shown that general irradiation of mice or rats with sub-lethal doses of X-rays increases and accelerates tumour production on subsequent treatment of the animals with a carcinogenic compound [Mayneord & Parsons, 1937; Clarkson *et al.* 1938; Parsons, 1938]. This change produced by the X-rays is one affecting the haemopoietic system: the red blood cells are diminished (in rats) and iron is deposited in the lymph glands.

Parsons and her fellow-workers have also shown that the same signs of blood destruction are present in mice undergoing treatment with a carcinogenic substance. Application of the prussian blue reaction to sections of the lymph glands of these animals before the appearance of tumours revealed the presence of iron. In a forthcoming publication the present author will adduce quantitative estimations of iron in support of these microscopical observations.

In view of these results it was considered of interest to carry out some simple *in vitro* experiments on the action of the compound used by Parsons and co-workers. The experiments were ultimately extended to embrace a series of similar compounds derived from carcinogenic and closely related non-carcinogenic hydrocarbons. The choice of the hydrocarbons to be used was necessarily restricted to those which yield adducts with maleic anhydride. For example, phenanthrene and 3:4-benzphenanthrene were excluded by this condition and, so far, all efforts to obtain an adduct from 3:4-benzpyrene have been unsuccessful.

Materials

The compound used by Parsons and co-workers was sodium 1:2:5:6-dibenzanthracene-9:10-*endo*-$\alpha\beta$-succinate [Cook, 1931]. Diels & Alder [e.g. 1931] and Clar [1932] have shown that hydrocarbons of the anthracene type form adducts with maleic anhydride. More recently Bachmann & Kloetzel [1938] have shown that the reaction is a balanced one and have described a procedure for obtaining good yields of the adducts from several important carcinogenic hydrocarbons.

In the present work the solutions of the Na salts of the *endo*succinic acids were prepared as follows. The anhydrides were prepared by boiling the hydrocarbon (1 part) in xylene or benzene (100 parts) with maleic anhydride (10 parts). After distillation of the solvent the anhydride was hydrolysed by warming for a few minutes with conc. KOH, the alkaline solution diluted with a large volume of water, warmed to effect solution of the K salt and filtered from unchanged

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hydrocarbon. On acidification of the filtrate the free acid was precipitated and collected. Solutions of the Na salts of the *endo*succinic acids were prepared by warming a slight excess of the acid with a measured volume of *N*/10 NaOH and filtering from the excess acid. Sufficient NaCl was added to the filtrate to make the final concentration 0·85 % (the effect of the *endo*succinate on the tonicity of the solution may be neglected since its concentration is low and its mol.wt. is high).

In a few cases this procedure had to be modified as the free acid on precipitation rapidly dehydrated to the anhydride which was insoluble in dilute NaOH. In these cases advantage was taken of the fact that the Na salts are very sparingly soluble in presence of NaCl and, after hydrolysing the anhydrides with conc. NaOH, the salt of the *endo*succinic acid was precipitated by saturating the solution with NaCl. The Na salt was collected and well washed with sat. NaCl and finally with 0·85 % NaCl to remove excess alkali.

The solubilities of the Na salts in 0·85 % NaCl vary considerably. In the group consisting of the derivatives of 1:2:5:6-dibenzanthracene (*cis* compound), 1:2:3:4-dibenzanthracene, 5:6-*cyclo*penteno-1:2-benzanthracene, 9:10-dimethyl-1:2-benzanthracene, cholanthrene and methylcholanthrene, the solubility in cold saline is not very great and the stock solutions consisted of saturated solutions of their salts. Anthracene, naphthacene, 1:2-benzanthracene and the remaining methyl-substituted 1:2-benzanthracenes employed gave Na salts which were relatively easily soluble and stock solutions of these were prepared containing 2 mg. per ml. (0·2 %). The *trans*-acid from 1:2:5:6-dibenzanthracene also gave a readily soluble Na salt.

In all cases the solutions used for the haemolytic tests had a *p*H of approximately 7·2.

The compounds used in this investigation are given in the following list under the name of the parent hydrocarbon. With one exception all the *endo*-succinic acids used have the normal (*cis*) configuration. Reference is made below to their method of preparation and in the case of six members of the series which have not been hitherto described, analytical specimens of the anhydrides were isolated and the results of their elementary analyses are given. All melting points are uncorrected.

Anthracene. Prepared from anthracene by the method of Diels & Alder [1931]. The Na salt is easily soluble in saline.

Naphthacene. The procedure given by Clar [1932] was followed. The Na salt is readily soluble.

1:2-Benzanthracene. Prepared according to Clar's directions. Na salt comparatively easily soluble.

1:2:5:6-Dibenzanthracene. The normal (*cis*) compound was prepared according to Bachmann's improved method. Conversion into the stereoisomeric *trans*-compound was effected in the manner described by Diels & Alder [1931] for the corresponding anthracene adduct. The *cis*-acid was converted into the *cis*-dimethyl ester [Bachmann & Kloetzel, 1938] and the latter rearranged by boiling in alcoholic solution with Na. The *trans*-ester which is formed as an intermediate is hydrolysed by the excess of $NaOC_2H_5$ to the *trans*-Na salt. The free *trans*-acid was liberated from the Na salt by acidification. A specimen of this acid on recrystallization from dilute acetic acid melted at 255–257° and gave a depression on mixing with a specimen of the pure *cis*-acid (M.P. 230°). The solubility of the Na salt of the *trans*-acid is very much greater than that of the *cis*-acid.

For further characterization a specimen of the *trans*-acid was converted into the dimethyl ester by means of diazomethane. The ester crystallized from

methyl alcohol in prisms, M.P. 179–180°. (The corresponding *cis*-ester has M.P. 230–231°). Found: C, 79·5; H, 5·6 %. $C_{28}H_{22}O_4$ requires: C, 79·6; H, 5·25 %.

1:2:3:4-Dibenzanthracene. The adduct was prepared in xylene. The xylene solution was cooled in the ice-chest overnight and the solid which separated was collected and recrystallized twice from xylene. Clusters of thick needles were obtained, M.P. 250–251°. Found: C, 82·85; H, 4·3 %. $C_{26}H_{16}O_3$ requires: C, 83·0; H, 4·3 %. The Na salt of the acid is sparingly soluble in saline.

5:6-cyclo-*Penteno-1:2-benzanthracene.* The free acid was prepared by the general procedure described above. On boiling a solution of the free acid in acetic anhydride for a short time and then cooling the solution the anhydride slowly crystallized in stout needles. After two recrystallizations from acetic anhydride the crystals melted at 245–246°. Found: C, 81·5; H, 5·0 %. $C_{25}H_{18}O_3$ requires: C, 81·7; H, 5·0 %. The Na salt of this acid is sparingly soluble in saline.

Cholanthrene. The adduct was prepared in benzene. The solid which separated on cooling the benzene solution was recrystallized from ethyl acetate. The anhydride crystallized in rectangular plates melting at 219–220°. Found: C, 81·5; H, 4·7 %. $C_{24}H_{16}O_3$ requires: C, 81·8; H, 4·6 %. The Na salt is sparingly soluble.

Methylcholanthrene. A specimen of the anhydride supplied by Dr W. E. Bachmann was converted into the Na salt of the acid. The salt is sparingly soluble.

9:10-Dimethyl-1:2-benzanthracene. A specimen of the anhydride was furnished by Dr Bachmann. The Na salt is sparingly soluble.

3-Methyl-1:2-benzanthracene. The free acid was prepared in the usual way. Treatment with acetic anhydride and recrystallization from the same solvent yielded the anhydride as prisms melting at 257–258°. Found: C, 80·9; H, 4·8 %. $C_{23}H_{16}O_3$ requires: C, 81·15; H, 4·7 %. The Na salt is relatively easily soluble.

5-Methyl-1:2-benzanthracene. Prepared as for the 3-methyl compound. The anhydride crystallized from acetic anhydride in fine needles melting at 252–253°. Found: C, 81·1; H, 4·8 %. $C_{23}H_{16}O_3$ requires: C, 81·15; H, 4·7 %. Na salt relatively easily soluble.

10-Methyl-1:2-benzanthracene. The anhydride prepared from the free acid by boiling with acetic anhydride crystallized from this solvent in prisms melting at 262–264°. Found: C, 81·0; H, 5·0 %. $C_{23}H_{16}O_3$ requires: C, 81·15; H, 4·7 %. The Na salt is relatively easily soluble.

Other methyl-substituted 1:2-benzanthracenes. Solutions of the sodium salts of the *endo*succinic acids of 4-, 6-, 2′-, and 3′-methyl-1:2-benzanthracenes and also of 2′:7-dimethyl-1:2-benzanthracene were prepared without isolating the intermediate anhydrides. The Na salts of these acids are all relatively easily soluble in 0·85 % NaCl.

Technique of the haemolysis experiments

Mice were killed by breaking the neck and the blood from the heart drawn off with a syringe as quickly as possible. The blood was discharged into a small volume of 0·85 % NaCl, centrifuged, the red cells washed twice with saline and suspended in a small volume of 0·85 % NaCl.

The aqueous solutions of the Na salts were set up in 4 small tubes; the first contained the "stock" concentration (2 mg. per ml. in the case of the more soluble salts and a saturated solution in the case of those salts which were not soluble to this extent), and the remaining 3 aliquot dilutions of the first. A fifth tube contained only 0·85 % NaCl as a control. To each of the 5 tubes two drops

of the red blood cell suspension were added and the tubes inverted once or twice to ensure mixing. The tubes were stoppered and observed at intervals for 24 hr. at room temperature.

In the control tubes the upper limit of the sinking red cells was quite sharply defined, and later these formed a compact deposit. In the experimental tubes the first sign of haemolysis was often a diffuseness in the upper limit of the falling red cells and the fluid above had a yellow tinge. Where the compound was very actively haemolytic, lysis occurred before any appreciable settling of the red cells had taken place and the first observable change was the increasing transparency as compared with the opaque suspensions in the control tubes.

Results

Using the simple technique outlined above the haemolytic actions of the 18 compounds previously mentioned were tested. Broadly speaking the activities of the compounds fall into three groups. In the 1st are those compounds which haemolysed rapidly and gave clear red transparent solutions within a matter of minutes. The members of this group are all sparingly soluble salts so it was not possible to arrange them in any order of activity as the saturated solutions employed were not necessarily of equal concentrations. In the 2nd group are those compounds which showed a lower degree of activity, and an approximate estimate of the relative potencies of these compounds could be obtained since the stock solutions of all of them contained 2 mg. per ml. In the 3rd group are three compounds which showed no evidence of haemolytic action even though left in contact with the red cells for 24 hr.

Table I gives a summary of the results for these three groups.

Table I

	Haemolytic activity
Group I. (Saturated solutions)	
*endo*Succinates from:	
1:2:5:6-Dibenzanthracene (*cis* and *trans*)	Strongly haemolytic
1:2:3:4-Dibenzanthracene	,, ,,
5:6-*cyclo*-Penteno-1:2-benzanthracene	,, ,,
Cholanthrene	,, ,,
Methylcholanthrene	,, ,,
9:10-Dimethyl-1:2-benzanthracene	,, ,,
Group II. (Solutions 0·2% and less)	
*endo*Succinates from:	
2′:7-Dimethyl-1:2-benzanthracene	+ + + + + +
5-Methyl-1:2-benzanthracene	+ + +
6-Methyl-1:2-benzanthracene	+ + +
10-Methyl-1:2-benzanthracene	+ + +
3′-Methyl-1:2-benzanthracene	+ + +
3-Methyl-1:2-benzanthracene	+ +
2′-Methyl-1:2-benzanthracene	+ +
4-Methyl-1:2-benzanthracene	+
Group III. (Solutions 0·2% and less)	
*endo*Succinates from:	
Anthracene	Inactive
Naphthacene	,,
1:2-Benzanthracene	,,

Discussion

The first point of interest in considering the results is any possible correlation between the haemolytic activities of the *endo*succinic acids and the known carcinogenic activities of the parent hydrocarbons. (It should be noted that the

water-soluble derivatives themselves have been tested for cancer-producing action in only three cases—the derivatives from 1:2:5:6-dibenzanthracene (*cis* and *trans*) and methylcholanthrene; all these produced sarcomas in mice.)

In Group I of the Table, 5 of the 6 hydrocarbons are potent carcinogens, the exception being 1:2:3:4-dibenzanthracene which in this laboratory produced no tumours when applied to the skin of a series of 20 mice, of which only 2 lived for more than a year, the last mouse dying on the 487th day. In an earlier experiment 3 epitheliomas were obtained in 3 out of 70 mice with an impure specimen of the hydrocarbon, the earliest tumour appearing after 619 days. No reports on this compound from other laboratories have been found in the literature. In Group II, the 2′:7-dimethyl-, 2′-methyl- and 3′-methyl-1:2-benzanthracenes have not yielded tumours in any experiments recorded as yet. The 5 remaining hydrocarbons in this group are definitely carcinogenic, but, with the exception of the 10-methyl derivative, inferior in carcinogenic potency to the active hydrocarbons of Group I. In Group III anthracene and naphthacene have not given tumours in any experiments as yet reported, while 1:2-benzanthracene produced 1 epithelioma when applied to the skin of 30 mice, although negative results have been obtained in another series of 50 mice.

In general it may be said, for the 18 compounds tested, that the water-soluble salt derived from a hydrocarbon which is known to be carcinogenic is haemolytic but that the converse is not necessarily true and hydrocarbons which have not been shown to be carcinogenic may give rise to haemolytic *endo*-succinic acids.

Summary

The Na salts of the *endo*succinic acids derived from 17 polycyclic hydrocarbons have been examined for haemolytic action on mouse red blood cells. Haemolytic action was shown by the water-soluble derivatives from all hydrocarbons with a pronounced carcinogenic activity. Haemolysis was also shown by the derivatives of some hydrocarbons which have not been found to be carcinogenic.

I am indebted to Dr L. D. Parsons, who suggested this investigation, for much help with the haemolysis experiments and to Dr W. E. Bachmann and to Prof. J. W. Cook for the gift of compounds. It is a pleasure to express my thanks to the Sir Halley Stewart Trust for a Fellowship held during this work and to the British Empire Cancer Campaign for generous grants which have supported this investigation.

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XX. *l*-(+)CITRULLINE

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(*Received 30 December 1938*)

Citrulline (α-amino-δ-carbamido-*n*-valeric acid) was first encountered as a constituent of the juice of the water-melon [Koga & Odaki, 1914; Wada, 1930]. Wada not only elucidated its constitution but produced it synthetically from ornithine. The substance was subsequently obtained by the action of putrefactive bacteria [Ackermann, 1931] and of *Bacillus pyocyaneus* [Horn, 1933] upon arginine, and also as a product of the tryptic digestion of casein [Wada, 1933]. More recently, methods have been described of preparing it in larger quantity from *dl*-ornithine [Kurtz, 1938] or from arginine [Fox, 1938].

Ackermann gives no information concerning the behaviour of his preparation towards polarized light, nor does Wada [1933] in the case of the product obtained from casein. All other specimens of citrulline hitherto prepared are described as optically inactive. The present paper describes for the first time the preparation of dextrorotatory citrulline and the determination of its specific rotatory power.[1]

l-(+)Citrulline was obtained simply by applying to *l*-(+)ornithine the excellent method lately devised by Kurtz [1938] for the preparation of *dl*-citrulline from *dl*-ornithine. This method consists in condensing the copper salt of ornithine with urea to form copper citrullinate. We have used it successfully with *l*-(+)ornithine hydrochloride prepared from gelatin by the enzymic method [Hunter, 1939].[2] An alternative procedure, when ornithine itself is not immediately available, is to apply Kurtz's method directly to the mixture of *l*-(+)ornithine and urea obtained by hydrolysing *l*-(+)arginine with arginase. For the success of this plan it is important that the hydrolysis of the arginine be complete, and that it be accomplished without introducing into the hydrolysate more than a minimum of extraneous material. The arginase solution used must therefore be at once purer and more potent than the ordinary crude glycerol extract of liver. One of us [Hunter, 1939] has recently described the preparation of a partly purified enzyme concentrate containing from 200 to 400 units [Hunter & Dauphinee, 1930] of arginase per ml. For the present purpose such a concentrate is entirely suitable.

The following account of one experiment among several shows the details of procedure adopted for the preparation of *l*-(+)citrulline from *l*-(+)arginine

[1] Since this paper was submitted for publication our attention has been drawn by M. Robert Duschinsky to his note (*C.R. Acad. Sci.* 1938, **207**, 735) in which are described the isolation of optically active citrulline from the water-melon and its synthesis by a method similar to our own. The figures given by M. Duschinsky for the rotation of the free amino-acid are in close agreement with those found by us.

[2] It is regretted that, in the discussion of the specific rotation of *l*-(+)ornithine dihydrochloride (p. 35), reference was not made to the work of Lutz & Jirgensons (*Ber. dtsch. chem. Ges.* 1931, **66**, 1221). These authors pointed out the variation of the specific rotation of the salt with concentration; they found $[\alpha]_D^{20°}$ +9·7° for $c = 1{\cdot}01$ and +13·3° for $c = 4{\cdot}1$.

hydrochloride. 10 g. of the latter salt were dissolved in 250 ml. of water, and enough *N* NaOH (3–4 ml.) was added to give a light pink colour with phenolphthalein. The solution was treated with concentrated arginase solution in such quantity as would supply 6000 units of the enzyme, and the mixture, adequately protected with toluene, was incubated at 37° for 18 hr.,[1] after which the Sakaguchi reaction for arginine was for all practical purposes negative. The mixture was then made acid to phenol red with acetic acid, boiled to coagulate proteins completely, filtered into a Claisen flask and evaporated *in vacuo* at 45–50° to about 50 ml. Frothing during the evaporation was controlled by addition of capryl alcohol.

The concentrated solution, containing equivalent quantities of ornithine hydrochloride and urea, was decolored with norite and filtered through a sintered glass filter. Flask and filter were washed with a few ml. of water, and the filtrate and washings brought, by dropwise addition of *N* NaOH, to *p*H just exceeding 7·0. The solution was then boiled, as described by Kurtz [1938], with 4 g. moist copper oxide, filtered, and, after addition of 10 g. urea, evaporated to 35 ml. After this concentrate, transferred to a tightly sealed pressure bottle, had been heated for 3 hr. in a boiling water bath, it yielded 7·4 g. of citrulline-copper (75 % of the theoretical).

The poor solubility of this product made impossible any observation of its behaviour towards polarized light; but, since it yielded a dextrorotatory citrulline (see below), and was derived from *l*-(+)ornithine, it must have been *l*-(+)*citrulline-copper*. In colour, in crystalline form and in relative insolubility in water, it did not differ in any obvious way from *dl*-citrulline-copper, as described by Wada [1930] and Kurtz [1938]. Recrystallization, which is difficult, was not attempted, and the material was probably not entirely pure. (Found: N (Kjeldahl) 19·8; Cu 15·3 %. $(C_6H_{12}O_3N_3)_2Cu$ requires N, 20·4; Cu, 15·44 %.) The M.P. was 249°. For the purified racemic compound Wada and Ackermann each give M.P. 257–258°.

l-(+)*Citrulline*. 5 g. of the copper complex were suspended in 125 ml. of water and decomposed with H_2S. Coagulation of colloidal copper sulphide was effected by heating with norite, and the latter was separated by centrifuging. The supernatant, still slightly coloured, was warmed again with a little norite, filtered through retentive paper into a Claisen flask, evaporated *in vacuo* to about 10 ml., transferred to an Erlenmeyer flask and treated with sufficient alcohol to produce a slight opalescence. Crystallization was started by rubbing with a glass rod, and was allowed to progress until, in about 20 min., a loose mass of crystals filled the entire liquid. Thereafter further additions of alcohol were made, about 10 ml. at a time, until during about 2 hr. 100–150 ml. had been introduced. After a night in the ice-box the crystals were collected on a glass filter, washed four or five times with 5 ml. portions of chilled alcohol and dried, first at 95° and then *in vacuo* over P_2O_5. The yield was 3·4 g., which is 80 % of the theoretical; the overall yield from the original arginine was therefore 60 %. (Found: N (Kjeldahl) 23·8 %. After recrystallization 23·9 %. $C_6H_{13}O_3N_3$ requires N, 23·99 %.) M.P. 215–217°; after recrystallization, 219·5°. For *dl*-citrulline Wada [1933] gives 226°, Ackermann [1931] 220–222°, Kurtz [1938] 220–221°. In form the crystals were long thin prisms similar to those figured by Wada [1933].

The rotatory power of the product was measured (1) in pure aqueous solution and (2) in the presence of 1 mol. HCl. For the measurements in water

[1] Experience has shown that the enzyme quantities recently prescribed by one of us [Hunter, 1939] were unnecessarily large, and the time of action unnecessarily long.

we used three different preparations, A, B and C; for those with acid, preparation B and two recrystallized mixtures of various origin. All measurements were made at approximately 5% concentration and at 21–23°. The results are collected in Table I.

Table I. *Specific rotation of* l-(+)*citrulline*

Sample	Mols. HCl	*l*	*c*	α	$[\alpha]_D^{22°}$ for $C_6H_{13}O_3N_3$	
A	0	2·2	5·005	+0·38°	+ 3·45°	+ 3·5°
B	0	2·2	5·025	+0·37°	+ 3·35°	
C	0	2·2	5·001	+0·40°	+ 3·64°	
B	1	2·2	5·016	+1·97°	+17·9°	+17·9°
Recrystallized (1)	1	2·2	4·974	+1·96°	+17·9°	
Recrystallized (2)	1	2·2	5·009	+1·98°	+18·0°	

It will be seen that the observed difference between any two preparations hardly exceeded the experimental error, and that the rotatory power was not altered by recrystallization. There is, therefore, no reason to suppose that the material was other than pure *l*-(+)citrulline. Its specific rotatory power in water is small—much smaller than that of the isomeric α-carbamido-δ-aminovaleric acid [Hunter, 1938]—but it is greatly increased by the presence of acid. In this respect citrulline resembles the related substances arginine and ornithine and indeed the amino-acids in general.

Summary

A procedure is described in which the method of Kurtz is applied to the preparation, from *l*-(+)ornithine or *l*-(+)arginine, of *l*-(+)citrulline. The yield from arginine is about 60%. In 5% aqueous solution the substance shows $[\alpha]_D$ +3·5°, increasing, in the presence of 1 mol. HCl, to +17·9°.

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XXI. STUDIES ON THE METABOLISM OF CALCIUM AND PHOSPHORUS AND ON THE AVAILABILITY OF THESE ELEMENTS FROM MILK AND FROM AN INORGANIC SOURCE

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(*Received 1 December 1938*)

Some time ago experiments were reported from this laboratory [Henry & Kon, 1937, 1] on the availability to rats of Ca and P in raw and pasteurized milks. While no difference was found in this respect between the two milks two rather puzzling observations were made. In the first place, though the rats received only 60 to 70% of the Ca and P normally retained by rats of their age on an adequate diet, they utilized only some 80% of their intake of these minerals. Secondly, 90% of the excreted Ca was found in the urine, a result hardly to be expected as it is usually believed that Ca is largely excreted in the faeces.

We have since carried out further experiments to determine whether this unusual excretion of Ca via the kidney and the rather low retention were due to the quality or to the quantity of the dietary Ca or possibly also to a relative shortage of P. At the same time we investigated the possibility of increasing the retention of P. It should be recalled that Ellis & Mitchell [1933] and Fairbanks & Mitchell [1936] had obtained almost 100% retentions of Ca from diets low in Ca but carrying a generous supply of P.

In these experiments the level, ratio and source of Ca and P were varied. Milk as a source of these elements was compared with Ca phosphate. Unless otherwise stated the general experimental methods were as described in detail by Henry & Kon [1937, 1].

Preliminary experiments

Experimental.[1] These were exploratory experiments which dealt mainly with factors affecting the assimilation of Ca. In one experiment Ca was supplied from milk and from $CaHPO_4$, $2H_2O$ at levels below the normal retention, in each case either with an equally subnormal level of P or with a more generous supply. In the second experiment the same plan was followed but all levels of intake of Ca and of P were raised.

1. *The diets.* To the basal low Ca and P diet of Henry & Kon [1937, 1] (diet 64, Table I) several supplements were added: (*a*) raw milk; (*b*) an equivalent quantity of diet 75, which was made from diet 64 by the addition of $CaHPO_4$, $2H_2O$ (Table I); 18 g. of diet 75 were calculated to be equivalent in calorie, Ca and P contents to 100 ml. of the raw milk; (*c*) raw milk + added P, derived from Na_2HPO_4, $12H_2O$; (*d*) diet 75 + added P from the same source. This extra P was calculated to cover the urinary and faecal loss of P observed in the previous experiments [Henry & Kon, 1937, 1], which amounted to

[1] Read in part before the Biochemical Society, 9 October 1937 [Henry & Kon, 1937, 2].

0·21 mg. P for each ml. of milk intake. The addition did not suffice to bring the intake of P to the level normally retained by rats (Table IV). The various supplements were fed separately from the basal diet, milk in the liquid form (with or without added Na phosphate) and diet 75 mixed to a paste with $\frac{3}{4}$ of its weight of water. When Na phosphate was added to it, the solution replaced part of the water. Table I gives the composition and analyses of the diets.

Table I. *Exps. 1 and 2. Composition and analyses of diets and milk*

	Diet 64	Diet 75	Milk*
Egg white†	14·25	13·86	—
Butter fat	5·00	4·86	—
Maize starch	68·40	66·56	—
Castor sugar	9·50	9·24	—
Ca- and P-free salt mixture‡	2·85	2·77	—
$CaHPO_4, 2H_2O$	—	2·71	—
% Ca	0·02243	0·69460	0·12540
% P	0·02585	0·54060	0·10700
% Moisture	9·96	10·20	—

* Average of 8 bi-weekly analyses. There was very little variation between samples. The Ca and P figures are per 100 ml.

† Dried, heat-coagulated from new laid eggs. ‡ Henry & Kon [1937, 1].

18 g. of diet 75 contained 0·1252 g. Ca and 0·0973 g. P. The small discrepancy in the P content of diet 75 and of the equivalent volume of milk is explained by the fact that the P in the milk was slightly higher than anticipated. The vitamin supplements were as described elsewhere [Henry & Kon, 1937, 1].

2. *The animals.* 4 groups of 4 litter-mate male rats were used. The rats were 26–27 days old and weighed 59–71 g. at the beginning of the experiment. Litter-mates within each group were assigned by lot to the 4 treatments. They were placed in glass metabolism cages [Henry & Kon, 1937, 1]. The intakes of diet 64 and of milk or diet 75 were equalized between groups (*a*) and (*b*) and between groups (*c*) and (*d*) respectively. Table II shows that in Exp. 1 the food intakes of all 4 groups were very similar.

3. *Exp. 1.* As in previous work [Henry & Kon, 1937, 1] the milk and diet 75 were fed at such levels that the intakes of Ca and P from them were well below the normal retentions as defined by Sherman & Macleod [1925] and Sherman & Quinn [1926]. As already mentioned, groups (*c*) and (*d*) received additional P from a 6·3 % solution of $Na_2HPO_4, 12H_2O$. After a preliminary period of 3 days on the experimental diets a collection period of 14 days was started. The excreta of each group of 4 rats were pooled for analysis.

4. *Exp. 2.* When Exp. 1 was finished the amounts of milk and diet 75 were so increased as slightly to exceed the calculated normal retentions. Groups (*c*) and (*d*) again received extra P from a 6·4 % solution of $Na_2HPO_4, 12H_2O$. Otherwise the details were the same as in Exp. 1. The preliminary period lasted 4 days and was followed by a 14 days' collection period. The same rats were used in both experiments.

5. *Results.* Table II gives the food intakes, weights of the rats and the weight of faeces. The mean balance figures for Ca and P are given in Table III. Further data will be found in Table IV.

(*a*) *Exp. 1.* In Exp. 1 the behaviour of rats receiving milk as a source of Ca and P (group (*a*)) entirely confirmed the results previously reported [Henry & Kon, 1937, 1] regarding the retention of these minerals and their paths of excretion on subnormal intakes. Again only about 80 % of the ingested Ca and P were retained and over 90 % of the excreted Ca was in the urine. When the

Table II. *Exps. 1 and 2 (14 days each). Food intakes, weights of rats and of faeces*

Group and source of Ca and P	Food intake: Diet 64 g.	Milk ml.	Diet 75 g.	Na_2HPO_4 solution ml.	Wt. of rats, g.: Initial	Final	Wt. of faeces‡ g.
			Exp. 1				
(*a*) Milk	106·13	164·99	—	—	67·5	107·7	2·49
(*b*) Diet 75	106·11	—	29·69	—	65·8	106·0	2·16
(*c*) Milk + Na_2HPO_4	108·68	165·57	—	6·619	67·5	109·2	2·62
(*d*) Diet 75 + Na_2HPO_4	108·68	—	29·75	6·614	67·8	107·8	2·31
			Exp. 2				
(*a*) Milk	107·32	293·48	—	—	117·0	150·8	3·80
(*b*) Diet 75	107·32	—	52·76	—	115·8	149·5	2·92
(*c*) Milk + Na_2HPO_4 (i)*	100·69	324·75	—	12·99	121·3	151·0	3·87
Milk + Na_2HPO_4 (ii)†	105·82	315·33	—	12·61	116·0	151·3	—
(*d*) Diet 75 + Na_2HPO_4 (i)*	101·00	—	58·34	12·96	119·3	153·8	2·84
Diet 75 + Na_2HPO_4 (ii)†	105·67	—	56·61	12·58	115·7	156·3	—

* (i) = Average for all 4 rats.
† (ii) = Average for 3 rats, omitting the rats mentioned in the text, p. 176.
‡ Air dry.

Table III. *Exps. 1 and 2 (14 days each). Mean balance figures for Ca and P*

Group	Ca intake g.	Ca excretion, g.: Urine	Faeces	Ca balance g.	P intake g.	P excretion, g.: Urine	Faeces	P balance g.
				Exp. 1				
(*a*)	0·2306	0·0486	0·0041	0·1779	0·2038	0·0129	0·0232	0·1677
(*b*)	0·2300	0·0691	0·0280	0·1329	0·1879	0·0040	0·0373	0·1466
(*c*)	0·2321	0·0315	0·0031	0·1976	0·2415	0·0351	0·0253	0·1811
(*d*)	0·2311	0·0369	0·0225	0·1717	0·2251	0·0240	0·0371	0·1640
				Exp. 2				
(*a*)	0·3934	0·0103	0·0145	0·3686	0·3420	0·0676	0·0302	0·2442
(*b*)	0·3906	0·0085	0·0380	0·3441	0·3129	0·0297	0·0550	0·2282
(*c*) (i)*	0·4317	0·0085	0·0170	0·4062	0·4468	0·1536	0·0356	0·2576
(*c*) (ii)*	0·4207	—	—	—	0·4352	—	—	—
(*d*) (i)*	0·4279	0·0052	0·0478	0·3749	0·4136	0·1119	0·0607	0·2410
(*d*) (ii)*	0·4169	—	—	—	0·4033	—	—	—

* Cf. footnotes, Table II.

minerals were derived from Ca phosphate (group (*b*)) the results were similar but the retentions (especially of Ca) were lower and more Ca was excreted in the faeces, reducing the urinary excretion to 71 % of the total. It should be remembered that the two groups (*a*) and (*b*) were not strictly comparable owing to the lower P intake of the latter group. Moreover, it is quite obvious from further experiments (pp. 176 and 184) that at low levels of intake the retention of Ca is markedly affected by the quantity of P in the diet. Further, milk and diet 75 differed with regard to buffering power and acid-base balance. These remarks apply equally to groups (*c*) and (*d*) and to all four groups in Exp. 2.

The addition of Na acid phosphate to the milk diet (group (*c*)) brought the P intake to 90 % of the normal retention and increased the retention of Ca to 85 % by considerably lowering the urinary excretion. The absolute retention of P also improved, though more was lost in the urine. The additional P had a similar effect on the rats receiving the Ca phosphate diet but the retentions of Ca (and of P), though increased, were still inferior to the retentions on the milk diet.

Table IV. *Exps. 1 and 2 (14 days each). Various data for Ca and P retentions and excretions*

Group	Ca: % Ca retention	Ca: % Ca intake derived from milk or diet 75	Ca: intake mg./g. gain in wt.	Ca: % of normal retention at same rate of intake*	Ca: % total Ca excretion in the urine	P: % P retention	P: % P intake derived from milk or diet 75	P: intake mg./g. gain in wt.	P: % of normal retention at same rate of intake†	P: % total P excretion in the urine
					Exp. 1					
(*a*)	77·14	89·7	5·74	72·1	92·31	82·30	86·5	5·07	78·4	35·83
(*b*)	57·77	89·6	5·72	71·9	71·12	78·01	85·6	4·67	71·1	9·74
(*c*)	85·12	88·7	5·56	70·9	91·09	74·97	73·4	5·79	89·3	58·17
(*d*)	74·28	89·4	5·78	72·2	62·14	72·85	71·5	5·63	86·5	39·32
					Exp. 2					
(*a*)	93·70	93·9	11·63	112·4	41·73	71·42	91·9	10·12	148·6	69·10
(*b*)	88·08	93·9	11·60	111·5	18·31	72·93	91·1	9·28	142·2	35·03
(*c*) (i)‡	94·09	94·8	14·53	139·3	33·40	57·67	78·0	15·04	223·4	81·18
(*c*) (ii)‡	—	—	11·91	116·9	—	—	—	12·33	185·2	—
(*d*) (i)‡	87·61	94·7	12·40	122·3	9·81	58·27	76·3	11·99	179·8	64·81
(*d*) (ii)‡	—	—	10·22	104·0	—	—	—	9·88	152·2	—

* Calculated from data of Sherman & Macleod [1925].
† Calculated from data of Sherman & Quinn [1926].
‡ Cf. footnotes, Table II.

(*b*) *Exp. 2.* In Exp. 2 it was aimed to supply to groups (*a*) and (*b*) quantities of Ca and P just above the normal retentions. Table IV shows that this was satisfactorily achieved for Ca in that the amounts fed surpassed the normal retention figures by about 12%, but that for P the margin was over 40%. This is explained by differences in the shape of the curves relating Ca and P retentions to the weight of the rat [Sherman & Macleod, 1925; Sherman & Quinn, 1926]. In groups (*c*) and (*d*) the Ca intakes were relatively higher (some 40 and 20% respectively above the normal) owing to the poor growth of one rat in each group. One rat in group (*c*) refused food during the last week and the intake of his litter-mate in group (*d*) had to be cut down accordingly. The marked discrepancy in the P intakes of these groups expressed as % normal retention (Table IV) was due to smaller gain in weight and to higher absolute P intake of one group. When the two unsatisfactory rats are omitted the average Ca and P intakes for the groups drop down to 117 and 185% and 104 and 152% respectively of the normal retentions.

The Ca intake in Exp. 2 was almost twice that in Exp. 1 (Table III), yet, in the presence of a relative surplus of P, the total excretion of Ca decreased because of a marked reduction in the urinary loss, so that more than twice as much Ca was retained and the efficiency of utilization rose to 94% for milk and 88% for Ca phosphate (Table IV). Further addition of P in groups (*c*) and (*d*) did not increase the % retention. There was a slight increase in the faecal loss of Ca in groups (*a*) and (*b*) in this experiment as compared with groups (*c*) and (*d*) of Exp. 1, but it should be pointed out that the shift in the % excretion of Ca from urine to faeces was chiefly due to the decrease of the urinary output.

It is probable that the retention of 94% of milk Ca was not maximal and that it might have been higher if Ca had been given at a level slightly below instead of above that of the calculated normal retentions. The increase in faecal Ca (Table III) supports this view as it indicates that excess unabsorbed Ca was passing through the gut.

The results of the two preliminary experiments throw light on some of the peculiarities noticed in our earlier work [Henry & Kon, 1937, 1]. They do not explain why P was not quantitatively retained, but the relatively low retention of Ca must have been due to an actual shortage of P as it was greatly improved by an increase in the level of P. The Ca of milk appears to be almost completely digestible and to be taken up in the blood stream. In the absence of sufficient P a part of it cannot be retained and is lost to the body through the urine. Hence much more Ca is excreted under these conditions through the kidney than through the gut. The Ca from an inorganic source behaves similarly but appears to be less digestible, since in all comparisons more was found in the faeces. Even then, under certain conditions, over 70% of it was excreted in the urine. On the Ca phosphate diets more P was also excreted in the faeces and when excretions on these diets are compared with excretions on the milk diets it is found that the excess faecal Ca and P are in the ratios of 1·70 : 1, 1·64 : 1, 0·95 : 1 and 1·23 : 1 respectively for the several milk-Ca phosphate comparisons (Table III), indicating the passage of unchanged $CaHPO_4$ through the gut. As already stated (p. 175) the milk and Ca phosphate diets were not strictly comparable. The problem of availability of Ca and P from different sources and the question of the metabolism of P were therefore further studied in the following experiments.

Experiment 3[1] (main experiment)

1. *The diets.* Two alterations were made in the composition of the diets. In the first instance liquid raw milk fed separately was replaced by spray-dried milk incorporated in the ration. This greatly facilitated the equalization of mineral intakes between milk and Ca phosphate diets which could be arranged beforehand with certainty. In addition it yielded information on the effect of drying on the availability of milk minerals. The second alteration was in the make-up of the substitute diet carrying the inorganic Ca phosphate. An attempt was made to render it equivalent to milk with respect not only to calorie, Ca and P contents but also to the relative proportions of constituents and to acid-base balance. A new diet was added to study the effect on the retention of P of excess Ca and of subnormal P intakes.

(*a*) *The basal diets.* The following three basal diets were used: (i) A diet low in both Ca and P, identical with diet 64 of the preliminary experiments (p. 173). (ii) A diet low in Ca but relatively high in P. It was made from diet 64 by adding to it sufficient $Na_2HPO_4, 12H_2O$ to raise the P content of the diet to 0·13–0·15%. This level was calculated from the preliminary experiments (p. 176) and from our previous work [Henry & Kon, 1937, 1] to supply, together with the milk or substitute diet, an excess of P over the normal retentions. (iii) A diet relatively high in Ca but low in P. This was obtained by adding $CaCO_3$ to the low Ca and P diet to bring the Ca content to 0·20–0·21%. Table V gives the analyses of these basal diets.

Table V. *Exp. 3. Ca, P and moisture contents of the basal diets*

Basal diet no.	% Ca	% P	% moisture
(i)	0·0195	0·0239	10·43
(ii)	0·0200	0·1489	10·91
(iii)	0·2170	0·0239	10·36

To each of these basal diets suboptimal amounts of Ca and P were added either from milk or from $CaHPO_4, 2H_2O$. This was done by mixing with the diets requisite quantities of spray-dried milk or of the substitute diet.

[1] Read in part before the Biochemical Society, 8 October 1938 [Henry & Kon, 1938].

(*b*) *The substitute diet.* The diet was planned to be equicaloric with the spray-dried milk, to have a similar acid-base balance and to resemble it regarding the protein, fat and lactose contents. As the Ca and P were to be given in inorganic combination as Ca phosphate, casein and lactalbumin were replaced by egg-white. Similarly the salts had to be altered. We are indebted to Dr W. L. Davies for advice on this matter. Information on the salt combinations in milk was taken from his book [Davies, 1936]. The composition of the diet is given in Table VI. It was prepared without the addition of $CaHPO_4$, $2H_2O$ and analysed for Ca and P. The calculated amount of Ca phosphate was blended directly with the basal diet and the rest of the substitute diet was then mixed in. For this reason Ca and P figures are not given in Table VI but Table VIII shows that the quantities of these elements in the substitute diet and in milk were practically identical.

(*c*) *The spray-dried milk.* The sample was dried by Messrs Aplin and Barrett at their Frome Factory in a Kestner plant after preheating. It was stored in sealed tins under N_2 and CO_2 and was 3 months old when used. The composition of the milk is given in Table VI.

Table VI. *Exp. 3. Composition of the substitute diet and of spray-dried milk*

	Substitute diet (as mixed) %	Dried milk %
Butter-fat	28·73	28·97
Egg white	27·63	—
Protein (N × 6·38)	—	25·94
Lactose	36·57	36·00
NaCl	0·402	—
KCl	0·342	—
K citrate	1·948	—
Mg citrate	0·689	—
$CaHPO_4.2H_2O$	3·682	—
Ca	—	0·9454
P	—	0·7307
Moisture	2·67	1·1

(*d*) *The final diets.* These were mixed in the proportion of 100 g. dried milk or 101·4 g. substitute diet to 569·56 g. of each basal diet. The slight difference

Table VII. *Exp. 3. Composition of the final diets*

Basal diets	(i) Low Ca and low P		(ii) Low Ca and high P		(iii) High Ca and low P	
Supplements	Milk	"$CaHPO_4$"	Milk	"$CaHPO_4$"	Milk	"$CaHPO_4$"
Final diet no.	89	90	91	92	93	94
Components:						
Egg white*	12·12	16·27	11·94	16·09	12·06	16·21
Butter fat	4·25	8·59	4·19	8·53	4·23	8·57
Maize starch	58·18	58·05	57·32	57·19	57·90	57·76
Castor sugar	8·08	8·06	7·96	7·94	8·04	8·02
Lactose	—	5·53	—	5·53	—	5·53
Ca- and P-free salt mixture†	2·424	2·419	2·389	2·383	2·412	2·407
NaCl	—	0·061	—	0·061	—	0·061
KCl	—	0·052	—	0·052	—	0·052
K citrate	—	0·295	—	0·295	—	0·295
Mg citrate	—	0·104	—	0·104	—	0·104
Na_2HPO_4, $12H_2O$	—	—	1·260	1·257	—	—
$CaCO_3$	—	—	—	—	0·423	0·422
Spray dried whole milk	14·94	—	14·94	—	14·94	—
$CaHPO_4$, $2H_2O$	—	0·557	—	0·557	—	0·557

* Dried, heat-coagulated from new laid eggs. † Henry & Kon [1937, 1].

in quantities between milk and substitute diets was due to difference in moisture content. The ratios in which the diets were mixed were calculated from the relative intakes of milk and basal diet observed in our previous experiments [cf. Table II and Henry & Kon, 1937, 1].

Table VII gives the composition and Table VIII the analyses of the final diets.

Table VIII. *Exp. 3. Analyses of experimental diets*

Diet no.	% Ca	% P	% moisture	Ca : P	% Ca derived from milk or Ca phosphate	% P derived from milk or Ca phosphate	% of norma retention supplied*	
							Ca	P
89	0·1610	0·1284	9·36	1·25	89·5	84·3	65·1	68·5
90	0·1610	0·1264	9·41	1·27	89·5	84·3		
91	0·1600	0·2367	9·95	0·68	89·3	46·3	63·8	125·5
92	0·1607	0·2328	10·01	0·68	89·3	46·3		
93	0·3284	0·1283	9·36	2·56	43·3	84·3	154·5	74·7
94	0·3280	0·1271	9·39	2·58	43·5	84·3		

* These figures were calculated after the experiment was completed from data of Sherman & Macleod [1925] and Sherman & Quinn [1926].

2. *The animals.* Six litters were used, each consisting of 6 male rats 23–24 days old and weighing 42–56 g. The animals were distributed by lot but in cases where differences in weight between litter-mates were appreciable uniform pairs were first selected for the milk-Ca phosphate comparisons and the final allocation was by toss of coin. The animals were numbered consecutively, rats 1–6, 7–12 etc. being litter-mates. The two types of metabolic cages used and the methods of feeding, giving vitamin additions, collecting excreta etc. were as already described [Henry & Kon, 1937, 1]. Distilled water was given for drinking. The experimental diets were fed for 4 days before the collection of excreta was started. The experiment lasted 5 weeks, the cages being washed once a week. At the end of the experiment the rats were killed by coal gas and the carcasses, after removal of the intestinal contents, were ashed and analysed for Ca and P. In ashing, 20 ml. of a 10% solution of Na acetate were added to supply fixed base, as without this precaution some loss of P occurred in our earlier experiments [Henry & Kon, 1937, 1].

3. *Results.* The results of this experiment are set out in Tables IX–XIII. Table IX gives the individual and mean data for the weights of rats and faeces; Tables X and XI give various data for individual rats concerning the intakes, excretions and retentions of Ca and of P. Table XII gives group means of data calculated from the two previous tables. Table XIII contains the results of statistical tests of significance of some of the differences. For this purpose the paired *t*-test of "Student" [1908; 1925] was used.

Towards the end of the experiment rat no. 34 on diet 92 showed a definite loss of appetite and passed blood in the faeces. During the last few days, therefore, no attempt was made to equalize the food intakes of this rat and of its litter-mate, no. 33, on diet 91. When the animals were killed for analysis it was found that the intestinal contents of rat 34 were, owing to the presence of blood, of a chocolate colour instead of the normal black. This was most probably due to internal injury caused by the animal nibbling off part of a metal tag on the cage. As can be seen from Table X the faeces of this animal contained abnormally large quantities of Ca and P, doubtless due to the haemorrhage. The figures for this rat (and for its litter-mates when necessary for comparative purposes) have been omitted from certain means.

Table IX. *Exp. 3 (35 days). Individual and mean data for the weights of rats and of faeces*

Rat no.	Wt. of rat, g. Initial	Final	Wt. of faeces* g.	Rat no.	Wt. of rat, g. Initial	Final	Wt. of faeces* g.
	Diet 89				Diet 90		
1	62	180	6·27	2	60	174	8·12
7	56	135	4·32	8	56	131	5·29
13	53	156	5·65	14	55	158	6·07
19	64	148	5·15	20	63	148	5·23
25	55	138	4·57	26	50	140	4·70
31	56	168	6·22	32	55	162	5·69
Mean (i)†	57·7	154·2	5·36		56·5	152·2	5·85
Mean (ii)‡	—	—	—		56·8	150·2	5·88
	Diet 91				Diet 92		
3	63	166	5·22	4	62	161	6·12
9	62	148	4·65	10	62	149	5·20
15	60	155	5·37	16	60	146	5·38
21	63	149	4·82	22	63	153	5·12
27	60	167	5·00	28	57	164	5·58
33	59	137	4·68	34	57	124	8·25
Mean (i)†	61·2	153·7	4·96		—	—	—
Mean (ii)‡	61·6	157·0	5·01		60·8	154·6	5·48
	Diet 93				Diet 94		
5	64	159	5·53	6	61	148	6·68
11	55	129	5·90	12	55	125	6·23
17	53	127	4·60	18	54	124	4·68
23	59	142	4·92	24	58	138	5·82
29	51	130	5·06	30	50	136	5·41
35	51	115	6·32	36	55	120	5·76
Mean (i)†	55·5	133·7	5·39		55·5	131·8	5·76
Mean (ii)‡	—	—	—		55·6	134·2	5·76

* Air-dry.
† Mean (i) = Mean for 6 animals.
‡ Mean (ii) = Mean for 5 animals, omitting litter VI (rats 31–36 incl.).

Table X. *Exp. 3 (35 days). Individual figures for the intakes and excretions of Ca and P*

Rat no.	Diet intake g.	Ca intake g.	Ca excretion, g. Urine	Faeces	P intake g.	P excretion, g. Urine	Faeces
			Diet 89				
1	377·93	0·6085	0·1293	0·0038	0·4853	0·0069	0·0577
7	286·88	0·4619	0·0763	0·0045	0·3684	0·0120	0·0341
13	359·96	0·5795	0·0901	0·0049	0·4622	0·0113	0·0459
19	332·99	0·5361	0·0888	0·0072	0·4276	0·0236	0·0462
25	306·27	0·4931	0·0795	0·0048	0·3933	0·0118	0·0399
31	386·46	0·6222	0·1175	0·0031	0·4962	0·0142	0·0534
			Diet 90				
2	377·70	0·6081	0·1541	0·0181	0·4774	0·0046	0·0842
8	288·00	0·4637	0·1008	0·0100	0·3640	0·0090	0·0528
14	359·59	0·5789	0·1068	0·0195	0·4545	0·0081	0·0582
20	331·17	0·5332	0·0860	0·0167	0·4186	0·0099	0·0562
26	306·07	0·4928	0·0946	0·0249	0·3869	0·0039	0·0498
32	385·33	0·6204	0·0971	0·0277	0·4871	0·0136	0·0553

Table X (*cont.*)

Rat no.	Diet intake g.	Ca intake g.	Ca excretion, g.		P intake g.	P excretion, g.	
			Urine	Faeces		Urine	Faeces
				Diet 91			
3	333·33	0·5333	0·0101	0·0027	0·7890	0·3039	0·0501
9	314·50	0·5032	0·0089	0·0027	0·7444	0·3029	0·0483
15	328·90	0·5262	0·0020	0·0038	0·7785	0·3065	0·0530
21	328·43	0·5255	0·0043	0·0037	0·7774	0·3299	0·0493
27	365·83	0·5853	0·0085	0·0036	0·8659	0·3673	0·0489
33	313·92	0·5023	0·0066	0·0041	0·7430	0·3329	0·0499
				Diet 92			
4	334·48	0·5375	0·0077	0·0116	0·7787	0·2991	0·0692
10	314·10	0·5048	0·0021	0·0119	0·7312	0·3018	0·0577
16	328·33	0·5276	0·0017	0·0163	0·7644	0·3189	0·0636
22	328·11	0·5273	0·0060	0·0174	0·7638	0·3142	0·0633
28	365·75	0·5878	0·0076	0·0116	0·8515	0·3443	0·0628
34	297·62	0·4783	0·0102	0·2082	0·6929	0·2543	0·1171
				Diet 93			
5	337·19	1·1073	0·4784	0·0993	0·4326	0·0048	0·0350
11	290·41	0·9537	0·2964	0·1931	0·3726	0·0045	0·0370
17	292·77	0·9615	0·2993	0·1646	0·3756	0·0052	0·0322
23	323·52	1·0624	0·3896	0·1222	0·4151	0·0063	0·0385
29	288·87	0·9486	0·3506	0·1235	0·3706	0·0056	0·0417
35	277·78	0·9122	0·2856	0·1852	0·3564	0·0056	0·0543
				Diet 94			
6	336·08	1·1023	0·4344	0·2035	0·4272	0·0062	0·0774
12	290·12	0·9516	0·3328	0·2330	0·3687	0·0054	0·0836
18	292·13	0·9582	0·3181	0·1800	0·3713	0·0067	0·0574
24	322·33	1·0572	0·3297	0·2475	0·4097	0·0056	0·0720
30	288·00	0·9446	0·3721	0·1942	0·3660	0·0044	0·0683
36	276·80	0·9079	0·4429	0·0956	0·3518	0·0038	0·0572

Table XI. *Exp. 3 (35 days). Individual figures for the Ca and P contents of the rat carcasses*

Rat no.	Wt. of carcass g.	Ca in carcass g.	Estimated deposited Ca, g.*	P in carcass g.	Estimated deposited P, g.*	Rat no.	Wt. of carcass g.	Ca in carcass g.	Estimated deposited Ca, g.*	P in carcass g.	Estimated deposited P, g.*
		Diet 89					Diet 90				
1	178	0·8346	0·4752	0·7243	0·4203	2	171	0·7624	0·4146	0·6764	0·3822
7	133	0·7471	0·4107	0·6151	0·3464	8	129	0·7084	0·3720	0·5972	0·3285
13	154	0·7572	0·4762	0·6504	0·4102	14	153	0·7591	0·4675	0·6453	0·3960
19	145	0·8297	0·4236	0·6653	0·3477	20	147	0·8128	0·4130	0·6590	0·3464
25	133	0·7377	0·4262	0·6082	0·3455	26	134	0·6935	0·4103	0·5790	0·3401
31	165	0·8808	0·5067	0·7337	0·4567	32	157	0·8410	0·4735	0·6922	0·4201
		Diet 91					Diet 92				
3	162	0·8840	—	0·7360	—	4	157	0·8793	—	0·7221	—
9	144	0·8685	—	0·6784	—	10	145	0·8588	—	0·6814	—
15	151	0·8412	—	0·6844	—	16	142	0·8251	—	0·6605	—
21	147	0·9165	—	0·7098	—	22	149	0·9045	—	0·6999	—
27	162	0·9161	—	0·7302	—	28	158	0·8914	—	0·7167	—
33	131	0·8858	—	0·6521	—	34	120	0·7909	—	0·5948	—
		Diet 93					Diet 94				
5	158	0·8765	0·5055	0·6868	0·3730	6	144	0·7883	0·4347	0·6247	0·3256
11	123	0·8084	0·4780	0·6112	0·3473	12	120	0·7047	0·3743	0·5500	0·2861
17	120	0·8015	0·5205	0·6000	0·3598	18	120	0·7494	0·4631	0·5693	0·3245
23	138	0·9123	0·5379	0·6724	0·3796	24	133	0·8350	0·4669	0·6345	0·3467
29	125	0·7757	0·4869	0·5790	0·3354	30	130	0·6492	0·3660	0·5458	0·3069
35	112	0·8146	0·4739	0·5845	0·3322	36	116	0·7558	0·3883	0·5711	0·2990

* Cf. p. 183.

Table XII. *Exp. 3 (35 days)*

(a) Group means calculated from data in Table X

Diet no.	Diet intake g.	Ca intake g.	Ca excretion, g. Urine	Ca excretion, g. Faeces	Ca balance g.	% Ca retention	% total Ca excretion in urine	P intake g.	P excretion, g. Urine	P excretion, g. Faeces	P balance g.	% P retention	% total P excretion in urine
89 (i)*	341·75	0·5502	0·0969	0·0047	0·4486	81·65	95·11	0·4388	0·0132	0·0462	0·3793	86·45	22·35
90 (i)*	341·31	0·5495	0·1066	0·0195	0·4235	77·06	84·29	0·4314	0·0082	0·0594	0·3638	84·34	12·32
90 (ii)*	332·51	0·5353	0·1085	0·0178	0·4090	76·49	85·58	0·4203	0·0071	0·0602	0·3529	84·03	10·84
91 (ii)*	334·20	0·5347	0·0068	0·0033	0·5246	98·12	62·82	0·7910	0·3221	0·0499	0·4190	52·98	86·52
92 (ii)*	334·16	0·5370	0·0050	0·0138	0·5182	96·50	25·91	0·7779	0·3157	0·0633	0·3989	51·25	83·27
91 (i)*	330·82	0·5293	0·0067	0·0035	0·5191	98·08	62·63	0·7830	0·3239	0·0499	0·4092	52·23	86·59
93 (i)*	301·76	0·9910	0·3500	0·1480	0·4930	49·75	69·77	0·3871	0·0053	0·0398	0·3420	88·22	12·01
94 (i)*	300·91	0·9870	0·3717	0·1923	0·4230	42·81	65·98	0·3825	0·0053	0·0693	0·3078	80·48	7·24
94 (ii)*	305·93	1·0028	0·3574	0·2117	0·4337	43·23	62·72	0·3886	0·0057	0·0717	0·3112	80·05	7·44

(b) Group means calculated from data in Table XI

Diet no.	Wt. of carcass g.	Ca in carcass g.	Ca in carcass %	Estimated† initial Ca, g.	Ca deposited g.	P in carcass g.	P in carcass %	Estimated† initial P, g.	P deposited g.	Ca : P ratio of carcass
89 (i)*	151·3	0·7979	0·5305	0·3448	0·4531	0·6662	0·4421	0·2784	0·3878	1·197
90 (i)*	148·5	0·7629	0·5162	0·3377	0·4252	0·6415	0·4336	0·2726	0·3689	1·190
90 (ii)*	146·8	0·7472	0·5123	0·3317	0·4155	0·6314	0·4321	0·2728	0·3586	1·186
91 (ii)*	153·2	0·8853	0·5790	0·3606	—	0·7078	0·4624	0·2888	—	1·250
92 (ii)*	150·2	0·8718	0·5809	0·3536	—	0·6961	0·4636	0·2972	—	1·252
91 (i)*	149·5	0·8854	0·5952	0·3662	—	0·6985	0·4683	0·2893	—	1·268
93 (i)*	129·3	0·8315	0·6481	0·3311	0·5005	0·6223	0·4840	0·2678	0·3545	1·338
94 (i)*	127·2	0·7471	0·5897	0·3315	0·4156	0·5826	0·4593	0·2678	0·3148	1·282
94 (ii)*	129·4	0·7453	0·5773	0·3243	0·4210	0·5849	0·4527	0·2669	0·3180	1·274

* Cf. footnote to Table IX.

† Cf. p. 183.

Table XIII. *Exp. 3. Statistical treatment of some of the differences*

Dietary difference tested*	Calcium: Difference	Calcium: Standard error of the mean	Calcium: P†	Phosphorus: Difference	Phosphorus: Standard error of the mean	Phosphorus: P†
			(*a*) % retention at different levels of Ca and P intake			
91–89	+16·44	±0·84	1 : over 11,000, signif.	—	—	—
92–90	+20·01	±1·64	1 : 3570, signif.	—	—	—
93–89	—	—	—	+1·77	±1·25	1 : 5, not signif.
94–90	—	—	—	−3·86	±0·93	1 : 111, signif.
			(*b*) % retention from milk or $CaHPO_4$			
89–90	+4·58	±1·15	1 : 96, signif.	+2·11	±0·95	1 : 13, not signif.
91–92	+1·62	±0·44	1 : 47, signif.	+1·73	±0·72	1 : 14, not signif.
93–94	+6·94	±0·88	1 : 1850, signif.	+7·74	±1·71	1 : 161, signif.
			(*c*) Faecal excretion from milk or $CaHPO_4$			
90–89	+0·0148	±0·0028	1 : 312, signif.	+0·0132	±0·0034	1 : 86, signif.
92–91	+0·0105	±0·0011	1 : 1430, signif.	+0·0134	±0·0017	1 : 735, signif.
94–93	+0·0443	±0·0314	1 : 5, not signif.	+0·0295	±0·0063	1 : 184, signif.
			(*d*) Urinary excretion from milk or $CaHPO_4$			
90–89	+0·0097	±0·0073	1 : 4, not signif.	−0·0050	±0·0020	1 : 18, not signif.
92–91	−0·0018	±0·0014	1 : 4, not signif.	−0·0064	±0·0061	1 : 3, not signif.
94–93	+0·0217	±0·0314	1 : 2, not signif.	0·0000	±0·0006	Not signif.

* The figures in this column refer to the numbers of the diets.

† P = probability that a mean difference at least as great as the observed mean difference would have arisen by random sampling from a homogeneous population.

The two diets containing additional Ca, nos. 93 and 94, appeared to be less palatable than the other diets. Less was eaten, there was a good deal of scattering and the animals grew less well on these diets (Tables IX, X and XII*a*). Though great care was taken in collecting the scattered diet as soon as possible, some contamination with urine could not be avoided. Such diet was then carefully dried by pressing between filter papers. These were then extracted with dilute HNO_3 and the washings were added to the urines. That this procedure was satisfactory and that the analytical results for groups 93 and 94 were correct can be shown in the following way. The animals receiving diets 91 and 92 did not scatter the food at all. It was therefore possible to calculate their initial stores of Ca and P from the analyses of their carcasses at the end of the experiment and from their Ca and P balances. These figures were then used to estimate [Henry & Kon, 1937, 1] the initial mineral stores of their litter-mates in groups 89 and 90, and 93 and 94. From these data and the carcass analyses the Ca and P depositions in these rats were calculated. Table XII *a* and *b* shows that the agreement between figures obtained in this way and the Ca and P balances of these rats is very good, especially as no litter-mates were actually analysed at the beginning of the experiment. These results justify confidence in the use of the urinary analyses for groups 93 and 94.

Turning now to a discussion of the results it is convenient to review them under two headings. The effects of changing the levels of Ca and P will be considered first and the differences in availability of Ca and P according to source will be dealt with later.

(*a*) *Effect of level of Ca and P in the diet.* When Ca and P were both supplied in quantities well below the normal retentions (diets 89 and 90) the results (Tables X and XII*a*) were very similar to those obtained in the previous experiments in this paper (p. 174) and in earlier work [Henry & Kon, 1937, 1].

Again the retention of Ca was about 80%, and some 90% of the excreted Ca was found in the urine. The retention of P, amounting to some 85%, was also not quantitative. In these respects it mattered little whether the minerals were derived from milk or from an inorganic source, there was no qualitative difference in their behaviour. It should be noted that there was a closer agreement between the milk and non-milk diets than in the preliminary experiments (p. 175), most probably because the two types of diets were this time more strictly comparable.

The most dramatic change followed the increase in dietary P (Tables X and XII*a*). This brought about a very marked increase in the retention of Ca, with the result that the % retention rose to 98·1 and 96·5 respectively for the two types of diets. Table XIII*a* shows the high statistical significance of this change. Our supposition that the originally low retention of Ca was due to a shortage of P was thus definitely confirmed. The excretion of Ca per g. Ca intake decreased almost tenfold. This was almost entirely due to a diminished excretion of Ca in the urine, the fall amounting to some 160–180 mg. per g. Ca intake or about 93–95% of the original urinary Ca. The faecal Ca also fell by some 30 and 27% respectively, but this amounted to only 3–10 mg. per g. Ca intake, as can be calculated from data given in Tables X and XII*a*. As a result a higher proportion of the small quantity of Ca still lost on these diets was found in the faeces; this is specially noticeable on the Ca phosphate diet. The increased retention of Ca was obviously accompanied by increased deposition of P, 567 mg. and 488 mg. of P respectively being deposited per g. increase in Ca deposition.

The increase in dietary Ca, the P being left unchanged (diets 93 and 94), had a much smaller effect on the metabolism of P. The % retention increased slightly but not significantly for the milk diet (Tables X, XII*a* and XIII*a*) but dropped significantly, though not to a marked extent, for the Ca phosphate diet. This latter effect was due to increased faecal loss (Tables X and XII*a*). In both cases the urinary excretion was diminished.

(*b*) *The availability of Ca and P from milk and from calcium phosphate.* In all three comparisons the rats retained more Ca and more P from milk than from the inorganic source (Tables X and XII*a*). The differences are more pronounced for Ca and Table XIII*b* shows that in every case the superiority of milk is statistically significant.[1] Under conditions leading to maximal retention of Ca (diets 91 and 92) the Ca from Ca phosphate is retained very efficiently, all but 3·5% being deposited in the body. This is only 1·6% less than the retention from milk and it is interesting that such a small difference should be statistically significant. This is probably because of the internal consistency of the results yielded by the two groups of rats, which can be calculated from Table X.

Of the differences in P retention only one, that between diets 93 and 94, is statistically significant; the others, though suggestive with odds of 13:1 and 14 : 1 against the results being due to chance, are not. The differences in availability of minerals between the milk and non-milk diets are mostly due to higher faecal excretions of both Ca and P on the Ca phosphate diet. These differences in faecal excretions are all statistically significant (Table XIII*c*) with the exception of the comparison of diets 93 and 94 in respect of Ca. The urinary excretions differ less and not always in the same direction. They are greater for

[1] Strictly speaking only 90% of the Ca was derived from milk or Ca phosphate. The remaining 10% from the basal diet was common to both treatments. A correction to this effect might be introduced in the calculations as has been done by Ellis & Mitchell [1933]. For reasons already given [Henry & Kon, 1937, 1] we prefer not to do this.

milk rats in one case for Ca (diets 91 and 92) and in two cases for P. Table XIII*d* shows that these differences are not significant. The faecal loss of both minerals is invariably higher on the Ca phosphate diet and the Ca : P ratio of the excess excretions suggests that the poorer availability of the inorganic additions was due to non-absorption of some of the Ca phosphate which passed unchanged through the gut. The ratios are 1·12 : 1, 0·78 : 1 and 1·50 : 1 respectively for the three comparisons. The Ca : P ratio of $CaHPO_4$, $2H_2O$ is 1·29 : 1.

Discussion

1. *The metabolism of Ca and P*

Most of the Ca excreted by adult animals on normal diets is found in the faeces and until recently it was commonly believed that the gut functions as an excretory organ for this element. It was thought that Ca was absorbed by the small intestine and re-excreted in the lower bowel. A summary of relevant work will be found in the reviews of Stewart & Percival [1928] and of Hunter [1930]. More recently, however, Nicolaysen [1934] and Christiansen [1936] came to the conclusion that much of the evidence brought forward in favour of an active excretion of Ca by the gut was either experimentally unsatisfactory or inconclusive. Their own experiments on dogs [Nicolaysen, 1934] or rats [Nicolaysen, 1937], and on rabbits and goats [Christiansen, 1936], did not support the view that the lower bowel excretes Ca. Recently also the careful observations of Welch *et al.* [1936] and of Johnson [1937] have shown that in man only negligible quantities of Ca are excreted by the colon. Wright *et al.* [1938] have come to a similar conclusion regarding the cat. On the other hand Cowell [1937] has produced experimental arguments for the excretion of Ca into the large intestine of the rabbit which cannot be lightly dismissed and Dr Quinn of the Onderstepoort Laboratory, who is studying the digestive processes of sheep by means of fistulae at several intestinal levels, is of the opinion that the possibility of active excretion of Ca in the colon of ruminants must be seriously considered [private communication]. It is quite possible that herbivora behave differently in this respect from omnivorous or carnivorous animals. For both the latter the recent evidence speaks strongly against the active participation of the colon in Ca metabolism and in our opinion our work on young growing rats lends definite support to this view. In the first instance we find that on diets supplying a sufficiency of P but not enough Ca for proper calcification, the latter mineral is most efficiently retained, the daily loss in the period of rapid growth amounting to only 0·2 mg. daily in the urine and 0·1 mg. in the faeces (Table XII*a*, diet 91). Under such conditions there can be no doubt that there is no significant excretion of Ca through the colon. But even when the supply of P is so reduced that the food Ca, though limited in amount, cannot be efficiently deposited in the bone and some of it must be excreted, this excretion takes place almost exclusively through the urinary path. The daily loss amounts in this case to 2·8 mg. in the urine but still to only 0·1 mg. in the faeces (Table XII*a*, diet 89). Admittedly these very low faecal excretions are found only on the diets in which milk supplies most of the Ca. When this is derived from Ca phosphate the daily losses of Ca in the faeces rise to 0·4 mg. (diet 92, Table XII*a*) and 0·6 mg. (diet 90, Table XII*a*) respectively, but the faecal P is correspondingly increased and, in all probability, the higher faecal Ca on these diets does not indicate any active excretion of Ca through the large intestine but only that a part of the Ca phosphate has passed through the gut unchanged. In this case the difference in the urinary loss of Ca is even more spectacular, 0·14 mg. daily for the diet

containing adequate P (diet 92, Table XII*a*) and more than 20 times this amount, 3·0 mg., daily for the low-P diet (diet 90, Table XII*a*).

The efficiency with which growing rats are able, under conditions favouring the uptake of Ca, to retain this element has already been pointed out by Ellis & Mitchell [1933]. These authors expressed the view that "in the growing rat and possibly in all growing animals there is no integral requirement of Ca for maintenance", and in the two preliminary reports on our present work [Henry & Kon, 1937, 2; 1938] we fully endorsed this view. Indeed, the retention by young rats of over 98 % of the ingested Ca, not in a short metabolic period but in the course of 5 weeks during which they almost trebled their initial weights, can hardly be otherwise interpreted. For reasons more fully set out below we believe that it would be difficult to improve on the retentions observed in our present studies. Strictly speaking, therefore, the daily loss of some 0·3 mg. Ca may represent the unavoidable minimum. Two-thirds of it are due to urinary leakage of circulating Ca and the rest probably represents the Ca of digestive secretions which have not been reabsorbed, and the Ca of bacterial bodies. According to Sherman & Macleod [1925] the intestinal tract of the rat contains only negligible quantities of Ca and therefore the contribution of the shed epithelium is of no consequence in this respect.

We have just shown (p. 184) that under certain conditions the loss of Ca was primarily due to a relative shortage of P. The cause of the P loss is, however, not so easily explained. It is clear that it could not be due in the first instance to a shortage of Ca and addition of Ca to the diet did not improve the retention. It appears, therefore, that even under optimal conditions on the milk diets a definitely greater loss of P than of Ca is unavoidable. The difference lies mainly in the magnitude of the faecal excretions: 1·1 mg. daily, or 10·4 % of the intake, for P (Table XII*a*, diet 93), as against 0·1 mg. daily, or 0·6 % of the intake, for Ca (Table XII*a*, diet 91). The urinary losses on these diets were very similar: 0·15 mg. daily or 1·4 % of intake for P and 0·2 mg. or 1·3 % respectively for Ca. It is possible that a part of the faecal loss represents P that is not available. The diets contained some 57 % of maize starch with a P content of 0·016 % [Henry & Kon, 1937, 1]. If we assume that 50 % of this P is phytin-P—a generous estimate [cf. McCance & Widdowson, 1935]—and that 75 % of it is not available to the rat [Lowe & Steenbock, 1936], we arrive at an estimate of about 12 mg. faecal P due to unabsorbable phytin. This is, however, not enough to explain the difference between the faecal excretions of Ca and P, as at least 30 mg. of faecal P are not allowed for. It may be pointed out here that Brooke & Smith [1933] observed in rats on a ration very deficient in Ca and low in P daily faecal excretions of 0·3 mg. Ca and 1·3 mg. P and that Nicolaysen [1937] found in Ca and P starvation corresponding figures of 0·45 mg. Ca and 1·0 mg. P. Using a diet containing 0·15 % Ca and 0·4 % P (of which 0·21 % was derived from K_2HPO_4) Rottensten [1938] noticed daily faecal excretions of 0·4 mg. Ca and 2·4 mg. P.

When allowance is made for the faecal P which may have been of phytin origin and, in the case of the diets containing $CaHPO_4, 2H_2O$, for the P from the unabsorbed Ca phosphate (cf. p. 185), there is evidence suggesting that the rest of the faecal P had been absorbed before it found its way into the gut. Thus, with diet 91, 99·4 % of the total Ca was absorbed (retained or excreted in the urine) which would leave practically the whole of the P in assimilable form. It seems therefore very probable that part of the faecal P was assimilated and then passed into the gut in the digestive juices and as shed epithelium. The findings of Johnson [1937] and Welch *et al.* [1936] on man, and Wright *et al.* [1938]

on the cat make it improbable that P is actively excreted in the large intestine. Intestinal epithelium doubtless contains much more P than Ca, but judging from the figures given by Johnson [1937] and Welch *et al.* [1936] for the P content of the material from isolated caeca, it is doubtful whether it makes an important contribution to faecal P. From the available recent analyses of digestive juices (cf. Baxter [1933] for mixed saliva, Gamble & McIver [1928], Ågren [1935], Ball [1930] and Walsh & Ivy [1928] for gastric and pancreatic juices: the latter, Drury [1924], Jones & Laing [1934] and Cheymol & Quinquaud [1937] for bile, and de Beer *et al.* [1935], Herrin [1935] and Wright *et al.* [1938] for *succus entericus*) it appears that they contain more Ca than P. Therefore, the mechanism for reabsorption of Ca must be much more efficient than that for P. It seems very probable that this is because a large part of the P present in the gut becomes fixed by the intestinal bacteria and is thus no longer available to the host. It has been shown by Osborne & Mendel [1914] that on low-residue diets like the diets used in the present study, bacterial bodies account for about 40% of the dry weight of rat faeces. P is a more essential component of bacteria than Ca, and though both vary within very wide limits according to bacterial species and to the composition of the medium, authorities agree that bacterial ash contains more of the former than of the latter [Topley & Wilson, 1936; Buchanan & Fulmer, 1928]. Barber [1931], who analysed many species, gives for various coliform organisms values for P of about 1·5% and for Ca around 0·2% of the dry weight. It would be idle to speculate as to the exact P content of the bacterial flora of rats, nevertheless, the following calculation yields very plausible figures.

Table XIV. *Faecal excretion of P per g. food intake, P intake or per g. faeces*

	Total solid intake g.	P intake g.	Faecal P mg.	Wt. of faeces g.	Faecal P/g. total solid intake		Faecal P/g. P intake		Faecal P/g. faeces	
					mg.	%*	g.	%*	mg.	%*
Raw and pasteurized milk†	367·89	0·5132	81·25	8·69	0·2209	100	0·1583	100	9·35	100
				Exp. 1						
Milk	129·03‡	0·2038	23·20	2·49	0·1798	81	0·1138	72	9·32	100
Milk + Na_2HPO_4	132·12‡	0·2415	25·30	2·62	0·1915	87	0·1048	66	9·66	103
				Exp. 2						
Milk	148·19‡	0·3420	30·20	3·80	0·2038	92	0·0833	53	7·95	85
Milk + Na_2HPO_4	146·67‡	0·4468	35·60	3·87	0·2427	110	0·0797	50	9·20	98
				Exp. 3						
Diet 89	341·75	0·4388	46·20	5·36	0·1352	61	0·1053	67	8·62	92
Diet 91	334·20	0·7910	49·90	5·01	0·1493	68	0·0631	40	9·96	107
Diet 93	301·76	0·3871	39·80	5·39	0·1319	60	0·1028	65	7·38	79

* Taking the figures for raw and pasteurized milk as 100.
† Henry & Kon [1937, 1].
‡ On the assumption that the milk contained 12·5% total solids.

Table IX shows that rats receiving diet 89 excreted on the average in the course of 5 weeks 5·36 g. of air-dry faeces. The faecal P output was 46·2 mg. (Table XII*a*) or 8·61 mg. P per g. faeces (Table XIV). On the assumption that 1 g. faeces contained 400 mg. bacteria [Osborne & Mendel, 1914] this works out at 2% P in bacterial bodies. A similar calculation for diet 91 gives 2·5% P and for diet 93, 1·8%; corresponding figures for Ca, omitting diet 93, are 0·22% and 0·16% respectively. The figures both for Ca and P are of the order quoted by Barber [1931] for various bacteria. If allowance were made for possible phytin-P not available to bacteria, the agreement with respect to P would be even better.

It seems therefore that as the faecal Ca on these diets can be entirely accounted for by Ca present in bacterial bodies the loss by this route could hardly be lowered. Moreover, the loss of 1·30 mg. Ca/g. faeces on raw milk diets (cf. Henry & Kon [1937, 1] and Exp. 1 of the present work) was greater than the figure of 0·77 mg. Ca/g. faeces obtained with the spray-dried milk diet in Exp. 3. It would appear therefore that drying, like pasteurization, has no untoward effect on the availability of milk-Ca for rats.

As a further argument in favour of the supposition that faecal P is mainly bacterial P, a comparison of all faecal P excretions on milk diets[1] shows that they agree much better when calculated per g. faeces than per unit of food intake or of P intake (Table XIV). A similar argument in favour of the bacterial origin of the faecal K has already been advanced by Peters [1933].

We are thus led to believe that there is no qualitative difference between the metabolism of Ca and that of P in the young growing rat. Neither Ca nor P can be adequately retained in the absence of sufficient quantities of P or Ca. When the available supply of the complementary element is adequate and that of the other is less than normal, only insignificant amounts of either Ca or P can leak out through the kidney.

The reabsorption of the quantities thrown into the gut with the digestive juices seems also to be equally efficient, the disparity in the amounts eventually lost not being due to any physiological or threshold differences but incidental to the symbiosis of host and intestinal flora. In neither case can we find any evidence of active excretion into the lower gut which, in the rat at any rate, does not in our opinion assume the functions of a vicarious kidney.

In our earlier experiments [Henry & Kon, 1937, 1] we found that when the supply of both Ca and P was inadequate, the requirements of the extra-skeletal structures for P were covered to a greater extent than those of the bones. Similar calculations applied to our present results yielded the values given in Table XV.

Table XV. *Exp. 3. Extent to which the requirements of the bones and extra-skeletal structures for P were satisfied by the rats receiving the experimental diets**

Diet no.	% normal P (and Ca) in bones	% normal P in tissues
89	65·7	85·6
90	64·1	85·1
91	71·7	85·3
92	72·7	84·9
93	85·3	77·8
94	78·6	76·5
Liquid milk†	61·0	82·7

* For method of calculation see Henry & Kon [1937, 1].
† Henry & Kon [1937, 1].

The results with diets 89 and 90, low in Ca and in P, agree very well with our previous findings. The addition of extra P (diets 91 and 92), and to a much greater extent the addition of extra Ca (diets 93 and 94), shift the P saturation from the soft tissues and blood to the bones which, when extra Ca is added,

[1] We have not carried out similar calculations for the diets containing Ca phosphate as they would be complicated by the presence in the gut of the unchanged compound (cf. pp. 177 and 185), especially as it was less well utilized in the preliminary experiments than in the final experiment. This can be easily seen by comparing the faecal excretions on milk and Ca phosphate diets in the three experiments.

appear to take precedence in their affinity for this element. It is possible that the inferior gain in weight of the rats on these diets may have had some effect on the result. It is of great interest, however, that Schneider & Steenbock [1938] have observed a similar effect in the presence of vitamin D on diets containing much more Ca than P.

2. The availability of Ca and P from milk and from inorganic sources

Experiments carried out on dogs some 20 years ago by Mendel and his collaborators [Givens & Mendel, 1917; McClugage & Mendel, 1918] showed that the Ca of milk was better utilized than that of Ca lactate or of $CaCO_3$. Previously, Aron & Frese [1908] had reported that the growing dog could obtain its Ca from Ca phosphate as efficiently as from milk. More recently Sherman & Campbell [1935; 1937] and Gaunt *et al.* [1938] showed that improvements in certain diets brought about by addition of milk could to a large extent be reproduced by the addition of $CaCO_3$ or of Ca lactate and Na_2HPO_4. Fairbanks & Mitchell [1936] found that the Ca of a diet containing 0·18 % of the element, mostly in the form of dicalcium phosphate, was completely utilized by young growing rats. Stearns & Jeans [1934] found that children retained Ca and P approximately as well from $CaHPO_4$ and $Ca_3(PO_4)_2$ as from milk. Finally Coward *et al.* [1938] came to the conclusion that the Ca of pasteurized milk is no more easily available for the growing rat than the Ca of inorganic salts (Ca phosphate plus Ca lactate). It has already been pointed out by one of us [Kon, 1938] and also by Gaunt *et al.* [1938] that this conclusion was not justified by the available experimental data.

As far as they go these studies appear to indicate that if there is a difference between the availabilities of Ca from milk and from inorganic sources it cannot be very marked and our metabolic experiments confirm such a view. We find that though spray-dried milk is a better source of Ca than $CaHPO_4$, the difference between them under conditions of optimal retention is small. It should be pointed out that we used commercial Ca phosphate and that though the analysis of the salt tallied very well with the formula, it is possible that it was chemically not quite pure.

Another point to remember is that the degree of saturation of the body stores of Ca has a marked effect on the avidity with which the mineral is deposited [Fairbanks & Mitchell, 1936; Rottensten, 1938] and it is possible that the gap between milk and Ca phosphate as sources of Ca would be different at other levels of intake.

Ellis & Mitchell [1933] found that pasteurization lowered the availability of milk-Ca. We [Henry & Kon, 1937, 1] have been unable to confirm this finding. It is of interest that the figure obtained by us now for the availability of the Ca of a diet containing dried milk—98 %—is the same as that found by Ellis & Mitchell [1933] for a diet containing raw liquid milk. Since then Fairbanks & Mitchell [1937] have reported that Ca from spray-dried skim milk was some 7 % less available than the Ca from raw liquid skim milk, while Fairbanks & Mitchell [1938] obtained an average retention of Ca of only 85 % on a diet containing this type of dried milk. Already Ellis & Mitchell [1933] had expressed the view that the lowering of the availability of Ca in milk by pasteurization was due to a partial destruction of vitamin C. To this Fairbanks & Mitchell [1937] have added now what appears to be a striking confirmation. In preliminary experiments in which dried milk was fed to rats as a source of Ca, with and without the addition of 1 mg. daily of crystalline vitamin C, they found with a high

degree of probability that the administration of ascorbic acid improved the utilization of Ca. Our present experience is against such a view and the problem is now under investigation in our laboratory.

Summary

1. The balance of Ca and of P derived either from milk or from $CaHPO_4, 2H_2O$ has been studied in three separate metabolic experiments on young male rats.

2. In all comparisons the Ca of milk was found to be more available than the Ca of $CaHPO_4, 2H_2O$.

3. In the presence of extra P (as $Na_2HPO_4, 12H_2O$) sub-optimal quantities of Ca were retained from spray-dried milk with an efficiency of 98·1 % and from $CaHPO_4, 2H_2O$ with an efficiency of 96·5 %. The difference between these two figures is significant.

4. Under such optimal conditions the daily loss of Ca is 0·3 mg. per rat. Of this one-third is lost in the faeces and two-thirds in the urine.

5. The retention of P is never so efficient, even in the presence of extra Ca (as $CaCO_3$). The minimum daily loss observed was 1·3 mg. per rat. Of this 1·1 mg., or nearly 90 %, was in the faeces.

6. It is suggested that this faecal P is mainly bacterial P and that the relatively inefficient retention of P at sub-optimal levels of intake is due to the fixation of some of this element by intestinal bacteria.

7. There is no evidence of active excretion of either Ca or P by the large intestine of the rat.

We are most grateful to Dr C. G. Anderson and to Prof. H. Raistrick for their helpful advice on the mineral constituents of bacteria and to Prof. Capstick for arranging the supply of the spray-dried milk.

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XXII. THE EFFECT OF CONTINUED TREATMENT WITH ANTERIOR PITUITARY EXTRACTS ON MILK VOLUME AND MILK-FAT PRODUCTION IN THE LACTATING COW

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(*Received 20 December 1939*)

In a previous paper [Folley & Young, 1938] it was shown that single injections into cows in declining lactation of crude preparations of prolactin and of thyrotropic hormone obtained from fresh ox anterior pituitary tissue, resulted in a substantial temporary increase in milk yield. On the other hand, a similar single injection of a prolactin preparation obtained from acetone-desiccated, commercial anterior lobe powder by a slight modification of the method of Bates & Riddle [1935], had no significant effect on the milk yield, although repeated daily injections of the same prolactin preparation caused a substantial increase in milk yield. In general it was found that in the cow the lactogenic action of the anterior lobe extracts used was paralleled by their glycotropic (anti-insulin) activity rather than by their prolactin (pigeon crop-gland stimulating) activity.

The present paper records the results of experiments in which the influence of repeated injections of prolactin and thyrotropic preparations with high glycotropic activity was investigated.

Methods

Animals. Thirteen dairy Shorthorn cows, belonging to the herd of the Reading University farm, were used in these experiments. Three groups, each of 4 animals, were selected so as to be as comparable as possible with regard to stage of lactation, time since mating if pregnant etc. A single cow was used in one experiment. The cows were allowed out to grass except when they came indoors for milking at approximately 8 a.m. and 4 p.m. each day. Their management was that usual for first-class dairy herds in this country.

Milk analyses. In addition to daily milk yield, percentages of fat and non-fatty solids in group-composite samples were determined twice daily throughout the experiment on the 3 main groups. The content of lactose, total chlorides and phosphatase was determined at intervals. The methods used for these determinations were those previously described [Folley & Young, 1938].

Anterior lobe extracts. Preparations "Prolactin-C" and "Thyrotropic-C" [Folley & Young, 1938] were used in this investigation. The method of preparation of these extracts and their general properties have already been described [Young, 1938, 1; Folley & Young, 1938]. In brief, the method of preparation consisted in the extraction of absolutely fresh ox anterior pituitary lobes with saline at *p*H 8 in the cold, followed by separation at 0° into the fractions soluble at *p*H 5·5 and those insoluble at this *p*H. The former constituted "Thyrotropic-C" and the latter "Prolactin-C".

Injection of animals. After a suitable control period, each of the 4 cows in Group A was given, on alternate days, an injection of 30 ml. of "Prolactin-C" equivalent to 10 g. of fresh anterior lobe tissue. Each cow in Group B (4 animals) received similar injections of "Thyrotropic-C". Group C served as a control group and each of these 4 animals was injected on alternate days with 30 ml. of a saline suspension of fresh ox liver equivalent to 10 g. of fresh tissue. These injections provided an adequate control for the possible influence on milk secretion of the introduction of non-specific protein into the circulation of lactating cows.

Repeated injections of "Prolactin-C", but not of the other tissue extracts used in the present investigation, produced local reactions of the type produced by injections of a crude saline extract of ox anterior lobe tissue [Folley & Young, 1938]. It was found that centrifuging the "Prolactin-C" preparation at *p*H 8 threw down material that was normally in suspension [cf. Young, 1938, 1] leaving a clear fluid. One cow received injections of 30 ml. of this fluid (equivalent to 10 g. of fresh tissue) on alternate days for 25 days and showed no local reactions.

Results

Milk yield. The curves for aggregate milk yield in the 3 main groups are shown in Fig. 1. The daily yield of the cows receiving 5 injections of Prolactin-C was very substantially increased during the period of injections. Although the

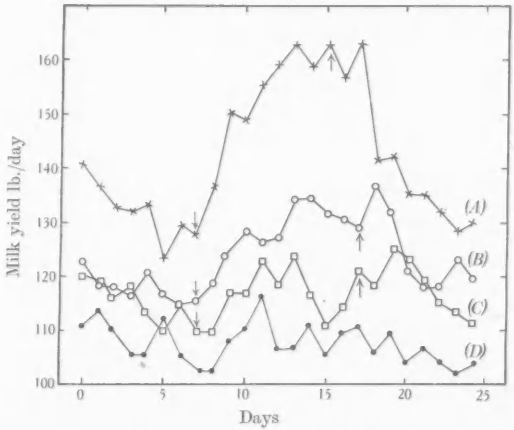

Fig. 1. Aggregate milk yields of groups of 4 cows receiving injections of pituitary extracts on alternate days. The amount of extract injected on each occasion was equivalent to 10 g. of fresh ox anterior lobe. ↓ signifies the first injection. ↑ signifies the last injection. Curve A: animals receiving Prolactin-C. Curve B: animals receiving Thyrotropic-C. Curve C: control animals receiving liver extract. Curve D: uninjected control animals.

milk yield did not continue to increase over the whole period of the injections, there was no sign of a decline from the highest level reached, until after the injections were discontinued.

Injections of Thyrotropic-C also increased the aggregate milk yield, although to a much less extent than those of Prolactin-C (Fig. 1), and again

there was no definite diminution of the increased milk yield until the injections had ceased.

In the group receiving liver extract a slight increase in milk yield occurred immediately after the initial injection. Unfortunately, on the day on which injections began it was found necessary to turn all the cows used in this experiment into a new pasture. In a normal year when grass is plentiful this would have made very little difference to the milk yield, but this experiment was carried out in the summer of 1938 when grass was very scarce owing to drought. Under these conditions a change to a new pasture might be expected to exert a stimulating effect on milk yield. That this was so is shown by the aggregate milk yield curve for the same period for a group of 4 untreated cows of the same herd (Fig. 1). The slight increase in milk yield which occurred following the injections of the liver extract cannot therefore be ascribed to a lactogenic action of this extract.

The milk yield curve for the cow which received injections of the centrifuged Prolactin-C is shown in Fig. 2. In this experiment a total of 12 injections was

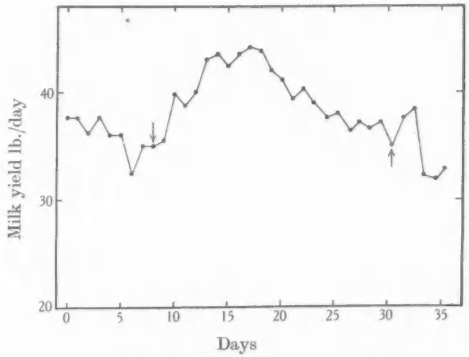

Fig. 2. Milk yield of a cow receiving injections of centrifuged Prolactin-C on alternate days. The amount of extract injected on each occasion was equivalent to 10 g. of fresh ox anterior lobe. ↓ signifies the first injection. ↑ signifies the last injection.

given, material being injected on alternate days. The milk yield rose to a maximum on the 10th day of the experiment, after which it steadily decreased, despite continued treatment.

Milk-fat percentage. The percentage of fat in the milk of the cows in Groups B and C (receiving injections of Thyrotropic-C and liver extract respectively) was unchanged during the experimental period (Fig. 3). The milk-fat percentage in the animals in Group A (receiving injections of Prolactin-C) was, on the other hand, very substantially increased as the result of the injections. As both the milk yield and the percentage of milk-fat were substantially raised, the total amount of fat secreted in the milk of these animals was very greatly increased as the result of treatment (Fig. 4). The total daily production of milk-fat by the cows in this group averaged 4·48 lb. for the 7 days before the injections were begun, and 6·63 lb. for the 10 days covering the period of the injections. Thus the daily production of milk-fat was increased by 48 %, on the average, following the institution of daily injections of Prolactin C.

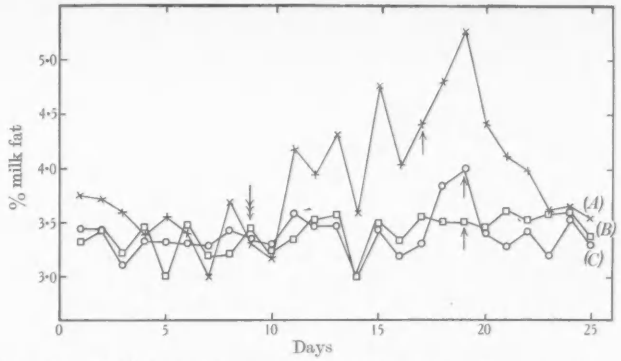

Fig. 3. Percentage milk-fat in pooled samples of the milk from groups of 4 cows receiving injections of pituitary extracts on alternate days. The amount of extract injected on each occasion was equivalent to 10 g. of fresh ox anterior lobe. ↓ signifies the first injection. ↑ signifies the last injection. Curve A: animals receiving Prolactin-C. Curve B: animals receiving Thyrotropic-C. Curve C: animals receiving liver extract.

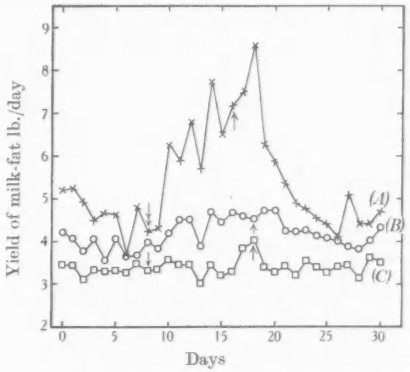

Fig. 4. Aggregate yields of milk-fat from groups of 4 cows receiving injections of pituitary extracts on alternate days. ↓ signifies the first injection. ↑ signifies the last injection. Curve A: animals receiving Prolactin-C. Curve B: animals receiving Thyrotropic-C. Curve C: animals receiving liver extract.

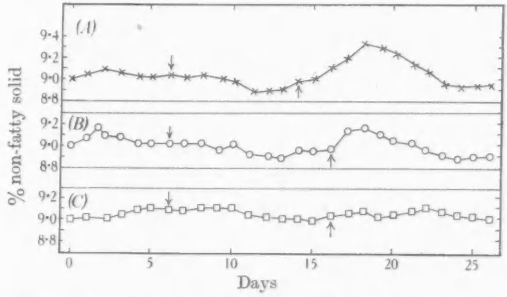

Fig. 5. Percentage non-fatty solid in pooled samples of the milk from groups of 4 cows receiving injections of pituitary extracts on alternate days. ↓ signifies the first injection. ↑ signifies the last injection. Curve A: animals receiving Prolactin-C. Curve B: animals receiving Thyrotropic-C. Curve C: animals receiving liver extract.

Non-fatty solid content of milk. The milk from the cows receiving injections of anterior lobe extract showed no clear indication of a significant change in non-fatty solid content (Fig. 5), there being a slight but probably insignificant fall. Lactose, chloride and phosphatase contents showed no regular alterations during treatment.

Discussion

These experiments show that the rate of milk secretion of cows in declining lactation can be increased by repeated injections of preparations of prolactin and of the thyrotropic substance, both possessing marked glycotropic activity, to a level much higher than was attained with single injections of these preparations. The single experiment in which 12 successive injections of prolactin were given showed that with prolonged treatment the milk yield reaches a maximum value and then declines, despite repeated injections. It does not seem probable that this decline was due to the production of an "antihormone" in the injected animal, as daily injections of ox-prolactin for 12–15 weeks were required to induce the appearance of substantial "anti-prolactin" activity in rabbits [Young, 1938, 2].

The great increase in milk-fat content as the result of treatment with prolactin was unexpected in view of the simultaneous increase in milk yield which occurred. The total production of milk-fat by each cow was increased by over 0·5 lb. a day as the result of this treatment. This increase is particularly interesting when it is remembered that the daily injection of a prolactin preparation relatively poor in glycotropic activity resulted in no significant increase in milk-fat content, although the milk yield was substantially increased thereby [Folley & Young, 1938]. There is no evidence whether or not the glycotropic substance influences fat metabolism, but it should be recalled that Marks & Young [1938] found that treatment of young rabbits with prolactin and thyrotropic preparations, both high in glycotropic activity, resulted in a substantial increase in liver fat. The glycogen content of the livers of these animals was also abnormally high. Similar results have been obtained with adult rabbits and with other species of animals (unpublished observations). It is not known at the present time how many active substances are present in anterior pituitary extracts of the type used in the present investigations. Until further fractionation of the active substances has been effected one can only attempt to correlate the observed physiological activities of the different fractions used. Table I summarizes the different physiological activities of the prolactin and thyrotropic preparations used in the present investigation. There is no clear indication of any correlation of the activity in increasing milk-fat content with any of the other activities investigated.

Table I. *Summary of physiological activities of different extracts used*

Physiological activity	Reference	Prolactin-C	Thyrotropic-C
Glycotropic (anti-insulin) activity	Young [1938, 1]; Marks & Young [1938]	+ + +	+ +
Increased liver fat content of fasting rabbits	Marks & Young [1938]; and unpublished data	+ + +	+ +
Increased liver glycogen content in fasting rabbits	Young [1937]; Marks & Young [1938]	+ +	+ + +
Milk yield increase in cows in declining lactation	Folley & Young [1938]; and present communication	+ + +	+ +
Increase in milk-fat content in cows in declining lactation (repeated injection of extract)	Present communication	+ + +	0

The injection of thyroxine into cows in declining lactation results in an immediate increase in milk yield, together with a delayed increase in the fat content of the milk [Folley & White, 1936]. The above results cannot be due to the thyrotropic activity of the pituitary extracts used, as the prolactin-C was most potent in influencing milk yield and fat content and this preparation has only a very slight thyrotropic activity [cf. Young, 1938, 1].

The results recorded in the present paper emphasize the undesirability of attempting to ascribe to any single substance of the pituitary gland stimulation of all the processes concerned with the secretion of milk. It is clear that in cows in declining lactation, neither the increase in milk yield nor the increase in milk-fat content, both of which are found to follow the repeated injection of a suitable pituitary extract, are related to the activity of the extract in causing growth of the pigeon crop-gland. The determination of how many different factors are involved in the control and stimulation of the complex processes of milk formation must await further fractionation of the extracts used.

Summary

1. Repeated injections of thyrotropic and prolactin preparations into cows in declining lactation resulted in a pronounced increase in milk yield, the increase being more marked with the prolactin preparation.

2. The increase in milk yield following injections of the prolactin preparation was accompanied by a substantial increase in milk-fat content. The average daily production of milk-fat was increased by nearly 50% during the period of 5 successive injections of prolactin.

3. Under prolonged treatment with prolactin the milk yield did not continue to increase, but reached a steady level and then declined, despite continued treatment.

4. The increase in milk yield and milk-fat content of cows in declining lactation, following the injection of pituitary extracts, cannot be ascribed to the action of a single substance.

We are indebted to Miss H. M. Scott Watson for valuable help with the calculation of results, and to Mr S. Watson for technical assistance. The co-operation of Prof. R. Rae and Mr K. W. D. Campbell of the University of Reading Farm, is gratefully acknowledged.

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XXIII. VITAMINS OF ELEPHANT'S MILK

By ZOFIA MARKUZE

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(*Received 29 December 1938*)

Following the birth of an elephant at the Warsaw Zoological Garden in June 1937 (the twelfth recorded case in Europe), a study of the chemical composition of elephant's milk was undertaken by Dr J. Królikowski of the Department of Genetics and Animal Dietetics of the School of Agriculture, Warsaw. The present paper describes the results of a comparison of the vitamin content of the milk, supplied to us by Dr Królikowski, with that of cow's milk. The samples of milk were taken periodically over the term of a year, commencing 8 weeks after parturition, one teat being milked while the calf was sucking another; the milk was delivered at the laboratory within an hour of milking. The staple food of the mother over the period consisted of grass, hay, wheat-bran, oil-cake, carrots and fodder beets. Prior to parturition, the fodder was supplemented with milk, and immediately after calving steamed linseed and wheat-bran were also given.

In view of the limited amount of milk available, the various vitamins were not determined simultaneously, but successively. This somewhat detracts from the value of the results obtained, since the relative contents of the individual vitamins may vary according to the season of the year, duration of lactation, nature of fodder and other variable factors. Raw cow's milk supplied by the Piaseczno Dairy was taken for comparison; the cows were fed on hay, grass, wheat-bran, oil-cake, soya bean, fodder beets and swedes. Studies were made of vitamins A, B_1, the B_2 group, C and D.

Experimental

Vitamin A. This was assayed by the curative method on rats given Mellanby's vitamin A-deficient diet [Green & Mellanby, 1928]. The increase in weight during a 4-week period of administration of milk was compared with that produced by the addition of 2–3 I.U. daily of vitamin A (International standard). The experiments were conducted in March and April (11th and 12th months of lactation). The results are given in Table I.

Table I. *Vitamin A*

Source of vitamin	Daily dose	No. of rats in group	Duration of experiment days	Mean wt. increase g.
International standard	2 I.U.	3	28	24
	3 ,,	3	28	37
Cow's milk	2 ml.	6	29	30
Elephant's milk	2 ,,	9	28	3
	2·5 ml.	4	28	5

The symptoms of xerophthalmia disappeared from rats given the International standard or 2 ml. of cow's milk daily, but not from those receiving elephant's milk. The latter animals did not increase in weight and at necropsy

exhibited the characteristic suppurative foci of avitaminosis-A. It may be concluded that the milk contained less than 1 I.U. of vitamin A per ml.

Vitamin B_1. This was assayed by the curative method on rats aged 3–4 weeks. Table II gives the average increase in weight of groups of rats maintained for 3 weeks on Evans' fat-free vitamin B_1-deficient diet [Evans & Lepkovsky, 1929], supplemented with varying amounts of milk, or with the International standard vitamin B_1 preparation (1 unit daily). The assays were carried out from September to November (5th to 7th months of lactation).

Table II. *Vitamin B_1*

Source of vitamin	Daily dose	No. of rats in group	Duration of experiment days	Mean weekly wt. increase g.
International standard	10 mg. (=1 I.U.)	6	21	10·0
Cow's milk	8 ml.	5	21	4·0
	10 ,,	4	21	5·0
Elephant's milk	3 ,,	5	21	7·5
	5 ,,	5	21	14·0
	8 ,,	5	21	14·5

It follows from the results obtained that elephant's milk contains $\leqslant 25$ I.U. of vitamin B_1 per 100 ml., compared with < 10 I.U. in cow's milk.

Vitamin B_2 complex. This complex was assayed on rats weighing 50–60 g. Their mean weight increases during 4 weeks on a diet deficient in B_2-vitamins [Sherman & Spohn, 1923], supplemented with 2 I.U. daily of vitamin B_1 and with 4 ml. daily of cow's or elephant's milk, are given in Table III. The assays were made from July to September (3rd to 5th month of lactation).

Table III. *Vitamin B_2 complex*

Source of vitamin	Daily dose	No. of rats in group	Duration of experiment days	Mean weekly wt. increase g.
Elephant's milk	4 ml.	5	28	10·7
Cow's milk	4 ,,	5	28	13·0

It may be concluded that the amounts of vitamin B_2 complex are approximately the same in cow's and elephant's milk.

Vitamin C. This was determined chemically according to the modification by Birch *et al.* [1933] of the method of Tillmans [1932]. Kon & Watson [1936] have shown that ascorbic acid is readily converted in the presence of light into dehydroascorbic acid, which does not react with Tillmans' reagent and which is reduced to ascorbic acid by H_2S. Reducing substances other than ascorbic acid were removed by the method of Emmerie and Van Eekelen [1934].

The ascorbic acid content of elephant's milk, tested in February (10th month of lactation), was 8·80 mg. per 100 ml. for skimmed, and 7·72 mg. for full cream, milk; the corresponding values for cow's milk were 2·18 and 2·12 mg. per 100 ml.

Vitamin D. This vitamin was assayed by the preventive method on rats receiving the rachitogenic diet No. 2965 of Steenbock & Black [1925], supplemented with 10, 12 or 15 ml. of elephant's milk daily, or with 1 I.U. of calciferol (international standard). The rats were sacrificed on the 30th day and epiphysial ossification was examined by the line test and by means of X-ray photographs. The rats receiving 10 and 12 ml. of milk had well-marked rickets, not differing in severity from vitamin D-deficient controls. Those given 15 ml. of milk daily

suffered from mild rickets, whilst those receiving 1 unit of international standard preparation showed normal ossification. Comparison with cow's milk was not performed in this case, but the author has found (unpublished) that 10 ml. of cow's milk contain about 1 I.U. of vitamin D. The assays were performed in November and December (7th and 8th months of lactation).

Summary

1. Elephant's milk has a lower content of fat-soluble vitamins (A and D) than has cow's milk.

2. Its vitamin B_1 content exceeds that of cow's milk, being 25 I.U. per 100 ml., as compared with 10 I.U. for the latter.

3. Elephant's and cow's milks have approximately the same content of vitamin B_2 complex.

4. Elephant's milk contains 7·72 mg. per 100 ml. of ascorbic acid, as compared with 2·12 mg. in cow's milk.

The author wishes to express her gratitude to Dr J. Zabiński, director of the Warsaw Zoological Gardens, for his co-operation, and to Dr R. Truszkowski for his helpful interest.

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XXIV. STUDIES IN THE STABILITY ON VITAMINS A AND D

II. ACTION ON FATTY PEROXIDES ON VITAMIN A

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(*Received 21 December 1938*)

The present widespread use of vitamin A in medicine calls for information as to its stability, particularly when dissolved in oils. Several workers [Huston *et al.* 1928; Evers, 1929; Norris & Church, 1930; Holmes *et al.* 1936] have studied the stability under different conditions of storage of vitamin A in fish liver oils and of vitamin A concentrates dissolved in vegetable oils. Wide variations in the rate of destruction of vitamin A have been recorded: Jones [1928] found a reasonable amount of vitamin A present in a malt extract and cod liver oil preparation 23 years old; at the other extreme Marcus [1931] found vitamin A to be largely destroyed in 3 hr. when dispersed on decolorizing charcoal. Other workers [Wokes & Willimott, 1927; MacWalter, 1934] have studied the rate of destruction of vitamin A in cod liver oil aerated at various temperatures. Dann [1932] investigated the rate of destruction of vitamin A in a cod liver oil concentrate dissolved in a range of solvents, on aeration at 98°, and found surprising variations from complete stability in certain alcohols and esters to rapid destruction in fatty acids. Fridericia [1924] and Powick [1925] found that admixture with rancid lard slowly destroyed vitamin A in cod liver oil, and suggested that the effect might be due to organic peroxides. Rosenheim & Webster [1926] found peroxides present in cod liver oil in which the vitamin A had disappeared on exposure to air and light. Wokes & Willimot [1927] suggested that the destruction of vitamin A caused by aerating cod liver oil was due to the formation of volatile organic peroxides, while Whipple [1936] attempted to correlate the peroxide value with the destruction of vitamin A in cod liver oil as rancidity developed. On the other hand, Griffiths *et al.* [1933] found that vitamin A was stable for at least 4 months in cod liver oil emulsions prepared with gum acacia containing peroxidase. Cady & Luck [1930] found that emulsification of cod liver oil with H_2O_2 only slowly destroyed the vitamin A present, and Dann [1932] found that vitamin A was stable in a cold alcoholic solution of H_2O_2 for 24 hr.

We have made a number of experiments on the stability of vitamin A (in the form of concentrates) dissolved at various strengths in various vegetable oils, with and without the addition of 0·05 % quinol as antoxidant. In all instances the vitamin A content (measured by the absorption at 328 mμ) diminished, sometimes to vanishing point, during the course of 2 years' storage, but the rates of destruction were extremely erratic, even to the extent that the same solution in different bottles deteriorated at different rates. In general, there was a tendency for the more dilute solutions (1200 and 12,000 I.U./g.) to deteriorate more rapidly than the stronger ones (60,000 I.U./g.). In some instances quinol

had a slight retarding effect on the destruction but in others it appeared inactive. This is contrary to the findings of Norris & Church [1930] and of various other workers who have used quinol to protect the vitamin A in solutions for animal feeding, but it must be noted that the solutions we tested were very much more concentrated. Many of the solutions were rancid at the end of the test, but vitamin A was still present in some of these rancid solutions, whereas little or none was present in some of the solutions which neither tasted nor smelt rancid. Nevertheless, it seemed that the most probable explanation of the erratic nature of these findings was that fatty peroxides were the agents immediately destructive of the vitamin A, since it is well known that the rate of development of peroxides in oils is affected by conditions of storage, the extent of exposure to air, and particularly by the amount of natural antoxidants present.

A number of samples of various vitamin A concentrates at a uniform strength of 12,000 I.U./g. in arachis oil stored for many months in partly filled bottles were available. These were examined both for the content of vitamin A and of peroxides (by a slight modification of Lea's method [1929]). It was found that always when the peroxide value exceeded 5, at least 70 % of the vitamin A had disappeared. All samples in which little destruction had occurred showed very low peroxide values, but nevertheless there were several samples with low peroxide values in which considerable destruction of vitamin A had occurred. It is, of course, possible that these samples had at some time contained more peroxides, which had been used up in destroying the vitamin A, or in oxidizing constituents of the oil.

A number of experiments were then made in order to test more rigorously the theory that fatty peroxides slowly oxidize vitamin A. In the first series, a number of samples of vitamin A in the form of rich fish liver oils and concentrates of different types were dissolved to a strength of 12,000 I.U./g. in a rancid olive oil having a peroxide value of 260.[1] The solutions were immediately transferred to a series of glass tubes, which were evacuated, filled with N_2 and sealed. The tubes were then heated in a boiling water bath, and at intervals a tube was removed and the vitamin A determined by measuring the absorption at 328 mμ. It was observed that a progressive bleaching of the solutions occurred, while the absorption at 328 mμ steadily diminished with time of heating. Further, the trough of the absorption curve on the short-wave side of 328 mμ became filled up as the reaction proceeded, until the later samples showed only general absorption. In every instance the actual absorption at 328 mμ was measured, irrespective of whether or not the curve showed a maximum at this wave-length. All values were corrected for the slight general absorption of the solvent oil at 328 mμ. Every experiment was done at least in duplicate. The results are shown graphically in Fig. 1. It will be observed that the curves fall into 2 groups. The curves corresponding with the more rapid destruction of vitamin A cover 3 samples of vitamin A concentrate, 2 from fish liver oil and 1 from mammalian liver. The curves corresponding with slower destruction of vitamin A cover two fish liver oils (one of them of exceptionally high potency). It has previously been shown [Bacharach & Smith, 1928] that vitamin A exists in fish liver oil in a state different from that in which it is present in concentrates, and it was suggested that vitamin A is an alcohol, which in fish liver oils is esterified with fatty acids. It is interesting to note that this difference is reflected in a greater resistance of the esterified vitamin A to oxidation by fatty peroxides. The matter was further tested by esterifying a vitamin A concentrate with stearic acid, and

[1] As defined by Lea; each g. of oil contained peroxide oxygen equivalent to 260 ml. of $N/500$ thiosulphate.

measuring the rate of destruction of the vitamin A by the rancid olive oil. The data are included in Fig. 1, and it will be seen that the points lie close to those for the fish liver oils.

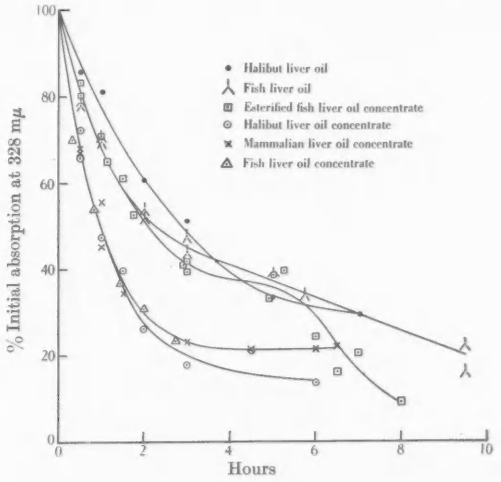

Fig. 1.

Fig. 1 shows clearly that the absorption at 328 mμ tends not towards zero, but towards a value of some 10–20 % of the initial absorption. The probable explanation of this phenomenon is that the primary reaction concerns only one of the double bonds in the vitamin A molecule, and that the primary oxidation product has an absorption band at a shorter wave-length than that due to vitamin A, but broad enough to show significant absorption at 328 mμ. Some confirmation of this was afforded by the $SbCl_3$ colour test. A solution in rancid oil after $8\frac{1}{4}$ hr. heating showed 11 % of the initial absorption at 328 mμ; the colour test indicated only 7 % of the initial vitamin A and, in addition, the test was abnormal; the solution became purple on adding the reagent, and the colour rapidly changed to blue, the reverse of the normal change. It is quite conceivable that some such colour reaction would be given by a primary oxidation product of vitamin A. There is some indication from the shape of the curves, in particular that for the artificially esterified vitamin A, that this primary oxidation product is itself slowly attacked by peroxide oxygen, with the production of a substance showing less absorption at 328 mμ. It is certainly possible for vitamin A to be oxidized to a substance almost completely transparent at 328 mμ, for we have had oil solutions decrease in absorption with development of rancidity to 1·5 % of the initial value, and this residual absorption was probably due to the glycerides.

In order to prove more definitely that the fatty peroxides themselves and not some other constituent of rancid oil were the actual destructive agents, a series of oils were prepared having different peroxide values. Olive oil was chosen as a typical vegetable oil and coconut olein as an oil mainly containing shorter carbon chains. Samples of these oils were exposed to the air in a warm place, while others were heated at 100° and others irradiated with a quartz

mercury vapour lamp. A sample of vitamin A concentrate was dissolved in each of the oils to a strength of 9600 I.U./g. and the samples were sealed in N_2 as before. They were all heated for the same times, namely, 2 and 4½ hr. at 100° and 45 hr. at 50°. The final vitamin A contents are shown in Table I, where the

Table I. *Fish liver oil concentrate dissolved in oils to 9600 I.U./g.*

Oil	Peroxide value ml. $N/500$ $Na_2S_2O_3$ per g.	% original $E^{1\%}_{1\,cm.}$ 328 mμ after 2 hr. at 100°	4½ hr. at 100°	45 hr. at 50°
Coconut olein V	0·5	96	—	95
Coconut olein VI	4·8	90	82	(87)
Olive oil I	10·5	83	81	94
Coconut olein VII	15	84	59	—
Olive oil II	18	79	65	96
Coconut olein VIII	36	74	57	87
Coconut olein IX	50	57	45	73
Olive oil III	64	57	39	73
Olive oil IV	125	41	22	63
Undiluted concentrate		94	92	96

oils are arranged in order of increasing peroxide value, irrespective of their nature (olive oil or coconut olein) or the method by which peroxide formation was induced. It will be observed that with very few exceptions this order is also the order of decreasing vitamin A content in the heated samples. The experiment was repeated with a sample of artificially esterified concentrate dissolved in some of these oils at a strength of 12,000 I.U./g. (Table II). No measurable destruction

Table II. *Esterified fish liver oil concentrate dissolved in oils to 12,000 I.U./g.*

Oil	Peroxide value ml. $N/500$ $Na_2S_2O_3$ per g.	% original $E^{1\%}_{1\,cm.}$ 328 mμ after 2 hr. at 100°	4½ hr. at 100°	100 hr. at 45°
Coconut olein V	0·5	100	100	—
Coconut olein VI	4·8	—	100	98
Olive oil I	10·5	100	100	100
Coconut olein VIII	36	91	88	88
Olive oil III	64	83	79	97
Olive oil IV	125	75	70	84

occurred, except with oils having a peroxide value of 36 or higher, but again (with one exception) increasing peroxide value went parallel with decreasing vitamin A; destruction was again much slower than with the unesterified concentrate.

No attempt was made to confirm these findings by biological assays, mainly because it was to be expected that the fatty peroxides would cause further destruction of vitamin A during the course of the assay or during any attempt to remove the peroxides, e.g. by saponification.

Some preliminary experiments along similar lines were done with solutions of calciferol in rancid olive oil, but it was impossible to assay the vitamin D accurately. Biological assays were contra-indicated, for the reason mentioned in connexion with vitamin A. There were indications that the oxidation products of vitamin D interfere with its direct spectrophotometric determination and even more with its determination by the Brockmann method with $SbCl_3$. Nevertheless, these experiments showed that calciferol is far more resistant than is vitamin A to destruction by fatty peroxides. Conditions that caused almost complete destruction of vitamin A caused barely 10 % destruction of vitamin D.

Discussion

In most of these experiments the concentration of peroxide oxygen was vastly in excess of that of the vitamin A, so that the reaction would be expected to follow a unimolecular, i.e. logarithmic law. When appropriate allowances were made for the absorption at 328 mμ of the first oxidation product postulated, the experimental results were found to follow approximately the logarithmic law.

In the second series it would be expected that the velocity of reaction would be proportional to the peroxide value. This was found to be the case within the limits of error of the spectroscopic determinations, except that the vitamin A in the solutions of low peroxide value was destroyed more rapidly than was to be expected. The indication here is that some factor other than peroxide oxygen caused very slow destruction of vitamin A. The fact that slow destruction of the undiluted vitamin A concentrate occurred when it was heated in N_2-filled tubes alongside the oil solutions suggests that this factor is simple heat. Edisbury *et al.* [1932] and Robinson [1938] have shown that heat can cause destruction of vitamin A in absence of O_2 or any oxidizing agent.

Conclusions

These experiments appear to afford clear evidence that fatty peroxides can cause destruction of vitamin A rapidly at 100° and more slowly at 50° and at room temperature. A practical consideration which follows is that the peroxide value of oil solutions of vitamin A needs to be considered, not merely as an index of potential rancidity, but as an index of the stability of the vitamin A. Moreover, a peroxide value not high enough to cause concern as to the stability of the oil itself may well be sufficient to cause slight destruction of dissolved vitamin A. The evidence that is here presented confirms that given in the previous paper of this series [Robinson, 1938] to the effect that oxidation products of vitamin A may show considerable absorption at 328 mμ. It follows that with vitamin A preparations that have suffered oxidation, a considerable portion of the absorption at 328 mμ may be due to substances other than vitamin A. Thus the present work affords further evidence that a spectrophotometric assay must be regarded with some suspicion when the band at 328 mμ shows poor persistence.

Fatty acid esters of vitamin A are more stable than the free alcohol towards fatty peroxides. This might be due either to the participation in the reaction of the hydroxyl group present in the free alcohol or, perhaps more likely, to activation by the hydroxyl group of the conjugated double bond system, rendering it more susceptible to attack by peroxide oxygen.

Summary

Vitamin A held in oil solution at room temperature disappeared at widely varying rates, but in general, destruction was greatest in solutions that developed a high peroxide value. Liver oils and concentrates were dissolved in rancid olive oil of peroxide value 260 and heated to 100° in N_2-filled sealed tubes. Progressive destruction of vitamin A occurred, more rapidly with the concentrates (of fish and mammalian origin) than with the fish liver oils (in which vitamin A occurs as an ester) or a re-esterified concentrate.

Oxidation of the vitamin A gave rise to a product with unselective absorption at 328 mμ, so that when oxidation has occurred the total absorption at 328 mμ indicates values in excess of the true vitamin A content.

Two concentrates were dissolved in samples of olive oil and coconut olein showing a wide range of peroxide values and the solutions sealed into tubes. After heating of the tubes under identical conditions, the extent to which the vitamin A had been destroyed was approximately proportional to the peroxide value and independent of the nature of the oil.

Grateful acknowledgements are made to Miss G. Adlington (Mrs White) and Miss B. E. Stern for assistance with the spectrophotometry, to Mr F. A. Robinson for helpful criticism, and to the Directors of Glaxo Laboratories Ltd. for permission to publish this paper.

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XXV. STUDIES IN THE STABILITY OF VITAMINS A AND D

III. THE EFFECT OF LIGHT ON VITAMIN A

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(*Received 21 December 1938*)

It is well known that the vitamin A in an oil or concentrate gradually disappears on aeration or storage. It has also been recorded that vitamin A is destroyed by irradiation [Chevallier & Choron, 1934; Chevallier, 1935; 1938; De, 1935; Morton & Heilbron, 1928; Morton *et al.* 1931; Zilva, 1919]. On the other hand, Boyle [1934] obtained a large increase in the value of $E^{1\%}_{1\,cm.}$ 328 mμ and in the Carr-Price value of a turbot-liver concentrate when this was irradiated for 20 min. Such a large increase has not been observed by other workers, though small increases in the value of $E^{1\%}_{1\,cm.}$ 328 mμ have been found to occur during storage of oils and concentrates for a short time, both by ourselves and by other workers [Gillam & Morton, 1931; Griffiths *et al.* 1933; Heilbron *et al.* 1931; MacWalter, 1934; Garratt, 1938]. The increase was in every instance slight and was always followed by a steady decrease. Chevallier & Choron [1934] claim that on irradiation of vitamin A a substance A^1 is first formed which is sparingly soluble in ethyl alcohol, but readily soluble in hexane, and has an absorption band with a maximum at 312·5 mμ; further irradiation results in the formation of β-ionone.

We have ourselves confirmed the findings of the majority of workers that a decrease in the value of $E^{1\%}_{1\,cm.}$ 328 mμ occurs when solutions of oils or concentrates containing vitamin A are irradiated, but we have encountered a new phenomenon, a brief report of which has already been published [Smith *et al.* 1938]. When a solution of vitamin A was irradiated and $E^{1\%}_{1\,cm.}$ 328 mμ was measured immediately, and again after standing in the dark for a few hr., it was found that the latter value was greater than the former. Furthermore, after a brief irradiation, causing a relatively small decrease in $E^{1\%}_{1\,cm.}$ 328 mμ, the value obtained after standing in the dark was found to be about the same as that of the unirradiated solution. More prolonged exposure to the irradiation was followed by a smaller degree of recovery on standing in the dark. As might be expected, the effect of irradiation was most marked with intense ultraviolet light, e.g. from a mercury vapour lamp, but a decrease in the value of $E^{1\%}_{1\,cm.}$ 328 mμ was also observed after several hours' exposure to daylight. In order to bring about an increase of $E^{1\%}_{1\,cm.}$ 328 mμ after intense irradiation it was not necessary for the irradiated solution to be kept in complete darkness; storage in daylight resulted in some recovery. Thus exposure to daylight brought about a decrease in $E^{1\%}_{1\,cm.}$ 328 mμ if the solution had previously been kept in the dark, but it resulted in an increase in $E^{1\%}_{1\,cm.}$ 328 mμ if the solution had previously been exposed to intense radiation.

Experimental

All absorption spectra were measured by means of a Hilger E2 Medium Quartz Spectrograph F_D 60 cm. with an H237 Spekker Photometer using tungsten-steel electrodes and a high-voltage spark. A range of exposures from

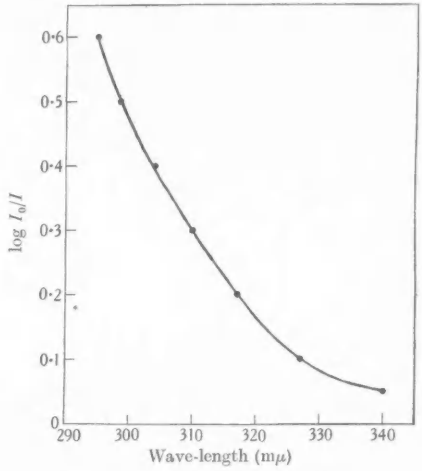

Fig. 1. Absorption of glass screen.

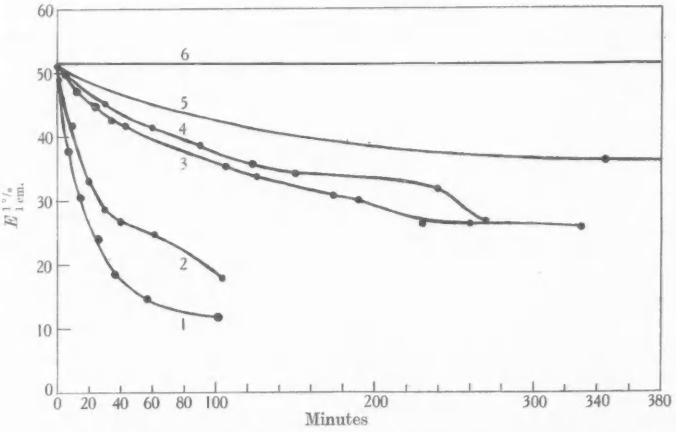

Fig. 2. Irradiation of fish liver oil No. 11/323. Curves 1–4 in cell with screen at following distances from mercury vapour lamp: (1) 20 cm.; (2) 26 cm.; (3) 35·5 cm.; (4) 45 cm. Curve 5, in diffused daylight, in specimen tube. Curve 6, in dark, in specimen tube.

0·5 to 25 sec. was normally used for each determination; the spectra were matched visually, and in every instance the accuracy of the match-points was checked by a second observer. The solution was transferred to the cell of the

spectrophotometer, and was irradiated either by exposure to the beam of light from the spark of the instrument, or by exposure to a mercury vapour lamp, after which the cell and contents were immediately transferred to the spectrophotometer for the absorption measurement. In order to minimize the effect due to light of very short wave-length, a glass microscope slide 1 mm. in thickness and having the transmission curve shown in Fig. 1 was interposed between the spark and the photometer in the first method and between the mercury vapour lamp and the cell in the second method. Fig. 2 shows a set of results obtained by irradiating an alcoholic solution of a fish liver oil with a mercury vapour lamp placed at different distances from the solution. It also shows the effect of

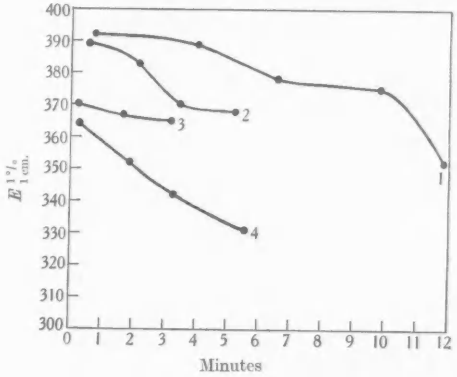

Fig. 3. Curve 1, irradiation of vitamin A concentrate No. 1/584 by tungsten-steel spark. Curves 2, 3 and 4, irradiation of same solution after recovery in the dark overnight.

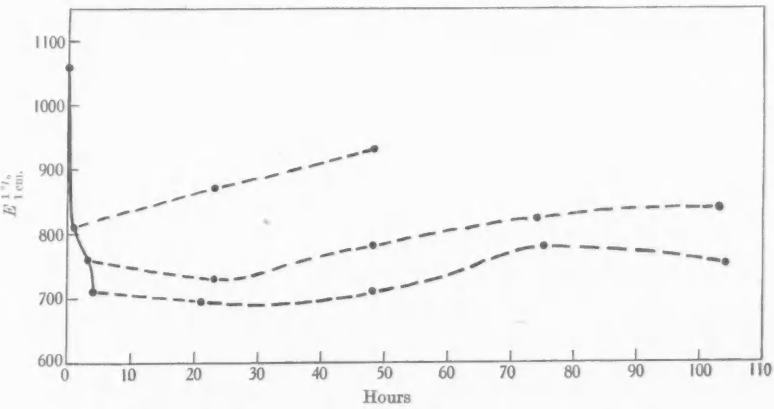

Fig. 4. Irradiation (—) and subsequent recovery (– – –) of concentrate No. S.A.I.

diffused daylight (from a north window) on the same solution. The same solution stored in the dark for over a month showed no appreciable change in the value of $E^{1\%}_{1\,cm.}$ 328 mμ. Fig. 3 shows a set of similar results obtained by

irradiating an alcoholic solution of a concentrate with the spectrophotometer spark; it shows also the recovery to almost the original value that occurred on standing overnight in the dark, and the further decrease in $E^{1\%}_{1\,cm.}$ 328 mμ that took place on re-irradiating. The amount of the subsequent recovery was very small.

Fig. 4 illustrates the effect of irradiating an alcoholic solution of a concentrate with a mercury vapour lamp and shows that the degree of recovery diminishes with the period of irradiation. Fig. 5 A shows similar results, a point of particular interest being that the concentrate showed a preliminary increase in the value of $E^{1\%}_{1\,cm.}$ 328 mμ when its solution was allowed to stand in the dark.

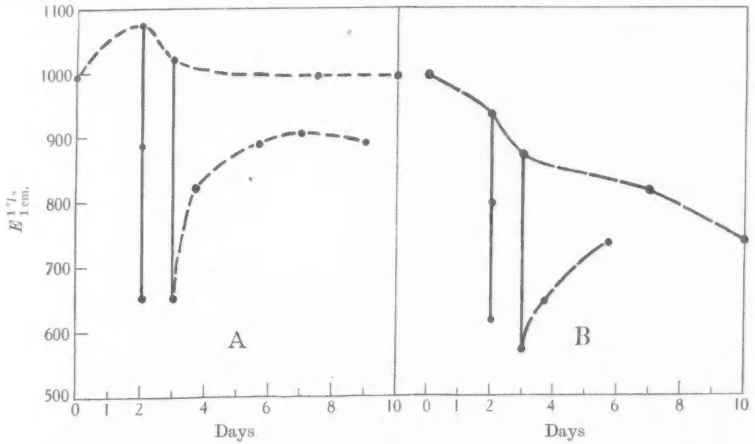

Fig. 5. Irradiation and recovery (A) in dark (B) in daylight of concentrate No. S.A.I. Irradiation ——; Daylight — — —; Dark – – –.

It is believed that previous exposure to daylight had reduced the value of $E^{1\%}_{1\,cm.}$ 328 mμ and that the reversible change had taken place when the concentrate had subsequently been dissolved in alcohol and allowed to stand. It is suggested that this is also the explanation of the slight increase in the values of $E^{1\%}_{1\,cm.}$ 328 mμ on storage of concentrates referred to above. Fig. 5 B illustrates the slow decrease in absorption of the same concentrate on exposure to daylight and the rapid further decrease on exposure to the mercury vapour lamp, followed by slow recovery in daylight.

Fig. 6 shows the effect of irradiation, under identical conditions, on solutions of a vitamin A concentrate of the same concentration in different solvents. For ease of comparison the ordinates represent percentages of the initial values of $E^{1\%}_{1\,cm.}$ 328 mμ, because these values varied slightly in accordance with the solvent effect noted previously [Smith *et al.* 1938]. The reduction in the value of $E^{1\%}_{1\,cm.}$ 328 mμ on irradiation is also not independent of the nature of the solvent.

The effect of daylight may be of analytical importance, because errors may be introduced by exposure of the solutions to daylight during saponification of oils and extraction of the soap solutions. Table I shows the reduction in ultraviolet absorption when vitamin A solutions were allowed to stand in the light for a few hours. With some spectrophotometers long exposure of the vitamin

solution to the light from the spark or arc occurs during measurement of the absorption spectrum, and this may also cause errors, but the effect is negligible when only short exposures are given.

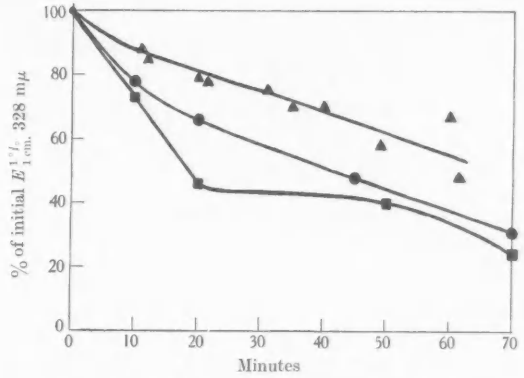

Fig. 6. Irradiation by mercury vapour lamp in various solvents.
■ Cyclohexane. • Ethyl alcohol. ▲ Ether.

Table I. *Keeping properties of vitamin A in daylight*

Kept in corked flask on bench by north-facing window

No.	Type of sample	Solvent	Month	$E^{1\%}_{1\,cm.}$ 328 mμ Initial	Final	Hr. exposure	% initial *E*
1/243	Concentrate	Cyclohexane	January	467	467	6	100
12/136	Halibut liver oil	Hexane	December	41·2	38·4	6½	93
12/168	Concentrate	Chloroform	January	405	387	7	96
S.A.I.	Concentrate	Cyclohexane	February	1030	821	47*	80
S.A.I.	Concentrate	Ether	February	1090	950	47*	87
S.A.I.	Concentrate	Chloroform	February	975	758	2	78
S.A.I.	Concentrate	Chloroform	February	960	915	2	95
					588	5	61
					85	22†	9
4/110	Concentrate	Cyclohexane	April	67·1	64·8	2	96·5
4/104	Concentrate (mammalian)	Cyclohexane	April	195	181	5¼	93

* Approximately 18 hr. of daylight. † Approximately 8 hr. of daylight.

Discussion

It is impossible at present fully to explain these experiments. The most plausible hypothesis appears to be that already advanced [Smith *et al.* 1938]. It seems likely that the vitamin A in an oil or concentrate exists as a mixture of geometrical isomerides, but that on irradiation energy is absorbed, causing a change in the proportion of isomerides. It is very probable, as pointed out by Morton [1938], that *cis-trans* isomerides of vitamin A would display different intensities of absorption. On removal from the radiation slow reversion to the equilibrium mixture occurs. It is evident that there is a simultaneous irreversible photochemical change of the vitamin A into some other substance, possibly bicyclic, with little absorption at 328 mμ.

SUMMARY

Exposure to ultraviolet radiations of wave-lengths exceeding 300 mμ approx. causes a progressive decrease in the value of $E^{1\%}_{1\,cm.}$ 328 mμ for vitamin A-containing concentrates or oils. This decrease is the more rapid the more intense the radiation, but even diffused daylight has some effect. When the irradiated solutions are allowed to stand in the dark (or in light of lower intensity) the value of $E^{1\%}_{1\,cm.}$ 328 mμ increases towards the original value. The further the value of $E^{1\%}_{1\,cm.}$ has been reduced by irradiation, the less complete is the recovery in the dark. It appears that vitamin A suffers reversible photochemical isomerization. Simultaneously slow photochemical destruction of the vitamin occurs.

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XXVI. PROLONGED ADMINISTRATION OF SEX HORMONES TO CASTRATED RATS

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From the Lister Institute, London

(*Received 23 December 1938*)

Our previous experiments with castrated male rats have been of only 23 days' duration [see references and technique, Korenchevsky, 1937; Hall & Korenchevsky, 1938, 2]. For obvious reasons the investigation of much longer periods of injections is important from both the experimental and the clinical viewpoints. In these experiments we have used the hormones which are known to occur naturally in males—androsterone, *trans*dehydroandrosterone and testosterone. Oestrone also belongs to this group of "natural" hormones, but in this investigation oestradiol dipropionate, a more active compound with a prolonged activity [Miescher *et al.* 1938] has been used. For the same reason the activity of testosterone propionate has also been investigated.

Technique

The hormones were administered for an average period of 105 days, the male hormones being injected 5 times a week, and oestradiol dipropionate 3 times a week, in the weekly doses indicated in Tables I and II. The total number of rats used for the experiments was 91, divided into 16 groups as shown in the Tables. The pure hormones were supplied by Messrs Ciba Ltd. and were dissolved in sesame oil, the single dose being injected in 0·2 ml. of the oil. The rats were castrated at the age of 22–25 days, i.e. before sexual maturity. The other details of our technique were the same as described in our previous papers.

Table I. *The effects of androsterone and* trans*dehydroandrosterone injected alone or simultaneously with oestradiol dipropionate on actual weights of organs of castrated rats*

	I	II	III	IV	V	VI	VII	VIII
					Androsterone 7·5 mg.		Dehydroandrosterone 7·5 mg.	
	Control rats (oil)		Oestradiol diprop.			+ 0·090 mg. oestrad. diprop.		+ 0·090 mg. oestrad. diprop.
	Normal	Castrated	0·018 mg.	0·090 mg.	Alone		Alone	
Seminal vesicles (mg.)	1309	12	131	129	403	153	39	74
Prostate (mg.)	1341	62	142	144	981	791	132	219
Penis (mg.)	364	98	132	140	289	210	158	163
Preputial glands (mg.)	128	38	65	47	149	117	133	84
Adrenals (mg.)	53	74	68	61	50	78	56	62
Hypophysis (mg.)	10·0	12·1	49·1	75·4	9·9	56·8	12·8	45·7
Thymus (mg.)	241	364	227	183	299	230	381	185
Liver (g.)	13·13	10·43	9·60	9·29	14·60	12·53	12·35	8·59
Kidneys (g.)	2·42	1·89	2·00	1·88	2·53	2·67	2·08	1·62
Spleen (mg.)	803	646	593	528	1022	863	973	607
Heart (mg.)	1135	950	868	833	1236	979	1066	785
Retroperitoneal fat (g.)	16	20	10	10	24	17	17	7
Final body weight (g.)	437	377	259	254	437	334	381	225
Gain in body weight (g.)	275	243	128	95	249	132	254	111
No. of rats in group	14	13	4	6	4	4	4	4

Table II. *The effects of testosterone and testosterone propionate injected alone or simultaneously with oestradiol dipropionate on actual weights of organs of castrated rats*

	IX	X	XI	XII	XIII	XIV	XV	XVI
	Testosterone 0·75 mg.		Testosterone 7·5 mg.		Testost. prop. 0·75 mg.		Testost. prop. 7·5 mg.	
	Alone	+ 0·018 mg. oestrad. diprop.	Alone	+ 0·090 mg. oestrad. diprop.	Alone	+ 0·018 mg. oestrad. diprop.	Alone	+ 0·090 mg. oestrad. diprop.
Seminal vesicles (mg.)	95	254	812	1216	1231	1097	2526	2363
Prostate (mg.)	217	405	720	960	1322	1161	2078	2087
Penis (mg.)	216	227	368	338	346	301	414	379
Preputial glands (mg.)	59	82	112	100	142	136	198	166
Adrenals (mg.)	56	75	58	56	57	84	62	77
Hypophysis (mg.)	13·7	30·1	11·9	64·5	11·7	36·1	9·6	21·0
Thymus (mg.)	275	165	234	107	220	203	95	54
Liver (g.)	10·62	10·94	11·07	8·69	12·95	10·56	11·15	8·80
Kidneys (g.)	1·87	2·31	1·98	1·78	2·35	2·41	2·59	3·19
Spleen (mg.)	870	805	805	666	952	855	803	755
Heart (mg.)	954	939	1016	740	1152	1021	1127	940
Retroperitoneal fat (g.)	13	11	12	9	17	11	9	5
Final body weight (g.)	339	281	348	239	419	337	335	255
Gain in body weight (g.)	229	175	208	97	275	185	177	82
No. of rats in group	4	4	4	4	6	5	7	4

For economy of space the weights per unit (200 g.) of body weight are not given, but they will be mentioned in the text whenever they alter the conclusion based on actual weights only.

Detailed histological results will be published elsewhere. In this paper are given only short preliminary statements without which the interpretation of the results would be difficult.

Oestradiol dipropionate will be abbreviated in the text to "oestradiol".

Effect on secondary sex organs

The effects of the prolonged injections on the seminal vesicles, prostate, coagulating glands, penis and preputial glands, atrophied after castration, were in some cases similar to, in others different from, those obtained in our previous experiments of shorter duration.

Male hormones. On the basis of the results of the present experiments the male hormones could be grouped in the following descending order of activity—testosterone propionate, testosterone, androsterone and dehydroandrosterone: only one hormone, testosterone propionate, was able to restore to normal (col. XIII), or, in large doses to produce supernormal (col. XV), development of seminal vesicles and prostate; the activity of androsterone was much more pronounced on the prostate than on the seminal vesicles (col. V). All these results were similar to those obtained in our 23-day experiments.

The prolongation of the injections strengthened the effect of androsterone, testosterone and testosterone propionate, but not that of dehydroandrosterone, which remained very weak, as comparison of the figures of Tables I and II with the data given in our previous papers [Korenchevsky *et al.* 1935–37] shows.

A close correlation was present between the degree of restoration towards normal of the weight and size of the organs and their histological structure. No pathological changes were observed, except for some abnormal vacuolation of the epithelial cells in the central and lateral lobes of the prostate in the rats injected with large doses of testosterone propionate.

Oestradiol dipropionate (cols. III and IV) increased the weight and size of the seminal vesicles to a degree remarkable for a female hormone, having a stronger effect on these organs than dehydroandrosterone. Even a definite hypertrophy of the penis was obtained, which has not previously been found with pure oestradiol or oestrone. The effect on the preputial glands, the weights of which vary considerably both in control and injected animals, was less definite. Increasing the dose from 0·018 mg. (col. III) to 0·090 mg. (col. IV) did not increase the effect on the male sex organs.

However, this remarkable effect of oestradiol on the weight and size of the seminal vesicles and prostate was not accompanied by restoration of the secretory epithelium, but was chiefly due to pathological development of fibrous tissue in these organs, while after shorter periods of injections [Korenchevsky & Dennison, 1935] the muscular layer was also greatly hypertrophied. The penis of the "oestradiol" rats showed better development of the corpora cavernosa and also partial separation of the epithelial layers of the preputium and of the glans penis, which are fused in castrated control rats. The epithelial layers showed slight development, but still resembled those in castrated control rats and not those in normal animals.

Co-operative or antagonistic activities of male and female hormones and the neutralizing effect of the male hormones on the pathological changes caused by the oestrogens

As judged by the weights of the organs, testosterone and oestradiol showed strong co-operative activity (cols. X and XII). This is in agreement with the results of our previous experiments of 23 days' duration [Korenchevsky *et al.* 1936]. This co-operative activity was confirmed histologically, especially with the large dose (col. XII); with the small dose of testosterone (col. X) the pathological effects of the oestradiol were not completely neutralized and were evident in the excessive development of fibrous tissue and stratification of the epithelium, which was, however, of the more normal columnar type. Testosterone propionate showed no co-operative activity with oestradiol (cols. XIV and XVI) in respect either of weight or histological structure; with both doses of testosterone propionate, the pathological effect of the oestradiol was seen only occasionally and in a slight degree.

The apparent absence of a co-operative activity of testosterone propionate with oestradiol is not due exclusively to the fact that the male hormone alone, in the doses used, produces normal or even supernormal development of the sex organs, so that its activity with the oestrogen is veiled. This conclusion is supported by the fact that in our previous experiments with small doses of testosterone propionate [Korenchevsky & Dennison, 1937] a similar result was obtained (only very slight effect on seminal vesicles). Slightly smaller sex organs in the rats injected with both hormones (cols. XIV and XVI) as compared with those injected with the male hormone alone (cols. XIII and XV) may be explained by the smaller size of the former rats; no such difference was seen in the weights of the organs calculated per unit of body weight.

An antagonistic effect was unexpectedly obtained when androsterone was injected simultaneously with oestradiol dipropionate (col. VI). This effect was very pronounced on the seminal vesicles (both in actual weights and those per unit of body weight), but less so on other sex organs (noticeable only in weights per unit of body weight). This antagonistic effect is the more remarkable since with smaller doses injected for shorter periods [Korenchevsky *et al.* 1935] a

definite co-operative effect of androsterone with oestrone was obtained both by weight and (unpublished data) histologically.

When *trans*dehydroandrosterone was injected simultaneously with oestradiol the weight of the seminal vesicles (74 mg. col. VIII) was less than that in rats injected with oestradiol alone (129 mg. col. IV). In this case, therefore, the male hormone exercised a definite antagonistic effect on the activity of the oestradiol. This interaction was not present in the case of the prostate and penis. Histological investigation confirmed this antagonistic activity of oestradiol with androsterone or dehydroandrosterone, at the same time revealing some neutralizing effect of these male hormones on the pathological changes produced by the oestrogen. Thus, although fibrous tissue was excessively developed and the epithelium was stratified (antagonistic "oestrogens" effect), the fibrosis was much less pronounced than with oestradiol alone, and the epithelium was of the more normal columnar type (neutralizing "male" effect).

Comparison with the effects of the same hormones on normal, non-castrated rats

In our experiments of long duration with non-castrated rats [Korenchevsky & Hall, 1939] it was unexpectedly found that the development of the secondary sex organs of these normal animals was depressed by androsterone, not significantly changed by testosterone and considerably stimulated by testosterone propionate. These remarkable differences in the activities of the male hormones were explained by the balance of two simultaneously exercised, opposite activities: (*a*) the depressing effect of the injected male hormones on the anterior lobe of the hypophysis, resulting in a more or less decreased secretion of gonadotropic hormones, which in turn decreases the secretion of the natural male hormones from the testes. This should produce atrophy of the secondary sex organs, but (*b*) this effect is counterbalanced by the stimulating effect produced directly, but in different degrees, by the injected male hormones on the secondary organs. In the case of the weaker sex hormones (androsterone and *trans*dehydroandrosterone) the atrophying effect (*a*) prevailed, but with testosterone propionate the hypertrophying effect (*b*) was the stronger. With testosterone, a hormone of medium strength, the two activities neutralized one another and therefore no change was obtained in the condition of the sex organs.

This mechanism cannot operate in the case of gonadectomized animals, and the results of our present experiments on castrated animals corroborate this: all the male hormones, without exception, produced stimulation of the secondary sex organs, the degree of stimulation produced corresponding to the strength of their sexual activity. The varying results obtained when the different male hormones were injected simultaneously with oestradiol probably depended upon the strength of the particular male hormone as compared with oestradiol. Therefore the different male hormones, when injected simultaneously with oestradiol into castrated animals, were divided according to their different degrees of activity into 3 groups, each consisting of the same hormones as in the case of normal (non-castrated) rats injected with the male hormones alone.

Effects on non-sexual organs, fat deposition and body weight

Effect of castration. We have described in our previous papers [Korenchevsky and co-workers, see references 1934–37] how castration produces more or less pronounced changes in the various non-sexual organs of the rat. The results obtained have been confirmed by our present experiments and may be summarized as follows (compare cols. I and II, Table I): varying degrees of hypertrophy of adrenals and hypophysis; delayed involution (i.e. increased size) of

the thymus; decrease in the weight of the liver, kidneys, heart and spleen (a definite change of the spleen recorded only in the present long-duration experiments); decreased growth and gain in body weight, but increased fat deposition; no definite changes in the thyroids. When calculated per unit of body weight, these changes are even more striking in the adrenals, hypophysis and thymus, but less pronounced in the other organs. Therefore a certain amount of the decrease in weight of liver, kidneys, spleen and heart corresponds to and can be explained by the smaller size and weight of the castrated animal. Part of this "oligosplanchnia" should, however, be explained by the specific effect of gonadectomy, i.e. by the absence of secretion of the testicular hormones. Specific histological changes after castration are well known and have been found in the hypophysis by several workers and in the adrenals by Deanesly [1928], Andersen & Kennedy [1933], Deanesly & Parkes [1937], Cramer & Horning [1937], Hall & Korenchevsky [1937; 1938, 1]. In the liver only a slight decrease in the size of the lobules has been observed [Hall & Korenchevsky, 1938, 2]. Histological examination of the other organs investigated in the present experiments has not yet been completed.

Effects of the hormones

Gain in body weight. The figures given in the Tables for the gain in body-weight during the experiment, taken in conjunction with the figures for the final body weight, strongly indicate an effect of the sex hormones upon metabolism. While oestradiol (cols. III and IV) produces a pronounced decrease in body weight resulting in stunted growth, with the male hormones this depressing effect is absent (androsterone, *trans*dehydroandrosterone, small doses of testosterone and testosterone propionate, cols. V, VII, IX, XIII) or less pronounced (large doses of testosterone and testosterone propionate cols. XI and XV). Moreover, this depressing effect of oestradiol can be partly neutralized by the simultaneous injection of male hormones (cols. VI, VIII, X, XIV) except in the cases of large doses of testosterone and testosterone propionate (cols. XII and XVI). These exceptions are interesting and important in that they demonstrate qualitatively different effects of different doses, which property is well known in pharmacology for several compounds and in endocrinology is especially important in the case of simultaneous injections of oestrogens and progesterone.

That the effect on the body weight is dependent upon the size of the dose is in most cases confirmed by comparison of the data of our present experiments with the results previously obtained. When injected for shorter periods (i.e. total dose being smaller), androsterone [Korenchevsky *et al.* 1935], testosterone [Korenchevsky *et al.* 1936] and small doses of testosterone propionate [Korenchevsky *et al.* 1937] caused increased gain in body weight, while large doses of testosterone propionate gave the same decrease, and dehydroandrosterone the same absence of effect both in long and short duration experiments.

A definite neutralizing property on the depressing effect of oestradiol is noteworthy in the weak sex hormones, androsterone and even to some extent dehydroandrosterone.

Fat deposition. With the exception of androsterone all the hormones investigated (including oestradiol) decreased, in varying degree, the amount of fat deposited. The depressing effect of oestradiol on the deposition of fat was considerably neutralized by simultaneous injections of androsterone, but not affected by small doses of testosterone and testosterone propionate, while dehydroandrosterone and large doses of testosterone and testosterone propionate when injected simultaneously with oestradiol caused an even greater loss of fat.

With shorter periods of injections (see references mentioned above), androsterone, dehydroandrosterone and testosterone produced no change in the fat deposition, while testosterone propionate decreased it as in the present prolonged experiments.

Adrenals. The hypertrophied "castration" adrenals (col. II), as judged by weight, both actual and per unit of body weight, returned to or towards normal (cols. V, VII, IX, XI, XIII and XV) after prolonged injections of male hormones, the changes both in weight and histologically being similar to those previously obtained in experiments of 3 weeks' duration [Hall & Korenchevsky, 1937; 1938, 1]. Large doses of testosterone propionate caused abnormal depletion of lipoid from some parts of the cortex. It is remarkable, however, that the longer period of injections did not, as one would theoretically expect, cause greater, but rather less pronounced, changes, as though some kind of neutralizing mechanism had developed during the prolonged administration of the male hormones.

When oestradiol was injected alone, the actual weight of the "castration" adrenal was slightly decreased but, when the weights are calculated per unit of body weight, a definite increase is observed with both doses (an average of 53 and 48 mg. in the injected rats as compared with 38 mg. in the control castrated animals). Evidently the weight of the adrenals was only slightly decreased in correlation with the stunted growth of the "oestradiol" rats, but the effect of the oestradiol on the adrenals is essentially hypertrophying. This conclusion is corroborated in a different way in most of the groups in which male and female hormones have been injected simultaneously (cols. VI, VIII, X, XIV, XVI): in these cases an abnormal hypertrophy of the adrenals occurred even in actual weights. A similar interrelation has been recorded in experiments of 3 weeks' duration [Korenchevsky and co-workers, 1935–37]. Previously also in experiments of shorter duration, Korenchevsky & Dennison [1934] found that oestrone produced hypertrophy in normal, but not in castrated, rats.

Histologically, oestradiol produced hyperaemia, a narrower *zona glomerulosa* with some atrophy of its cells, patches of peculiar amyloid-like degeneration in the *zona reticularis*, and the appearance of "nests" of specific cells at the boundary between the cortex and the medulla.

These "oestradiol" changes in the adrenals were not completely neutralized by the simultaneous injection of male hormones, thus corroborating the results obtained by weights.

Hypophysis. It is now well established that oestrogens produce enlargement of the anterior lobe of the hypophysis, resulting, with sufficiently large doses, in a tumour-like hyperplasia of the gland. In our present experiments we also have obtained similar changes in weight (Table I, cols. III and IV) and size of the hypophysis.

Male hormones alone, in both our previous and present experiments and in those of other workers, had no considerable or constant effect on the size and weight of the hypophysis. Allanson [1937] has given a good review of the literature on the subject and added her own histological results.

In our previous experiments of 3 weeks' duration, the weight of the "castration" hypophysis was decreased to normal only after injections of androsterone. In the present long-duration experiments this recovery was effected by androsterone (col. V) and also by large doses of testosterone propionate (col. XV).

It is necessary to emphasize the most important result that all the male hormones used were able to prevent to some extent development of the hypophysial hypertrophy caused by oestradiol, the greatest effect being produced

by the large dose of testosterone propionate (col. XVI, average weight 21 mg. as compared with 75·4 mg. in the rats injected with oestradiol alone). In one of the rats of this group (testosterone propionate + oestradiol) the hypophysis weighed 14 mg., which weight is within the variations present in the control uninjected groups.

Wolfe & Hamilton [1937], in experiments of 10 days' duration, also noticed some suppression of oestrogenic changes in the hypophysis by male hormones. Thus a definite antagonistic interrelation between male and female hormones is apparent in their effects on the hypophysis.

Thymus. All the hormones, except dehydroandrosterone, hastened the involution of the thymus, and the simultaneous injection of oestradiol and male hormones had a co-operative effect, these results being similar to those obtained in our shorter-duration experiments (see references already mentioned).

Liver, kidneys, spleen and heart. From a study of the data given in Tables I and II, it is possible to come to a general conclusion that male hormones cause an increase, and oestradiol (except in kidneys) a decrease, in the weights of these organs in most rats. Dehydroandrosterone and large doses of testosterone and testosterone propionate appear to increase this "oligosplanchnic" effect of oestradiol. Androsterone, on the contrary, is able to neutralize this effect, even when large doses of oestradiol are given, while in the cases of testosterone and testosterone propionate this neutralization occurs only with small doses of oestradiol. However, for true megalo- or oligo-splanchnia, not only the actual weights, but also the weights per unit of body weight should show the same change, i.e. the changes in weight of the organs should be at least partly independent of the changes in weight and size of the animals. If the changes we have obtained are considered from this point of view, and the weights per unit of body weight (not included in the Tables) are also taken into consideration, the following conclusions are reached:

(1) The male hormones can be considered as true stimulators of certain organs in castrated rats, but not in normal animals [cf. Korenchevsky & Hall, 1939]. In other words, they restore the size and weight of the liver, kidneys, spleen and heart to or towards normal, but are unable to cause supernormal enlargement.

(2) It is remarkable that this stimulating activity should be most strongly manifested, not by the most sexually active hormone, testosterone propionate, but by the relatively weak sex hormone, androsterone.

(3) The female hormone, oestradiol dipropionate, can be regarded as a stimulator only when the weights are calculated per unit of body weight (e.g. these weights of liver, kidneys and heart in rats injected with oestradiol were respectively 7·4, 1·5 and 0·66 g. as compared with 5·5, 1·0 and 0·49 g. in castrated control rats).

(4) Since the body fat in the "oestradiol" rats is much decreased, a comparison with the much fatter control animals is difficult. From the point of view of metabolism, one unit of passive fat tissue requires much less activity of the organs regulating metabolism (liver, kidneys, endocrines etc.) than do the more active tissues (e.g. muscular, glandular, nervous etc.). Therefore in fat animals one would expect that the weights of these "metabolism" organs, when calculated per unit of body weight, should be smaller than in animals in which the amount of body fat is small, provided that the metabolic rate is about the same.

According to Reed *et al.* [1930] the amount of genital and retroperitoneal fat in rats represents about 32% of the whole adipose tissue in the body. According to our observations, the amounts of these two types of fat (as

dissected by us) are about equal (i.e. 16%). If in all groups of our rats the total body fat is calculated on this basis and subtracted from the final body weight, the remaining weight will represent, at least chiefly, the approximate weight of the active tissues in the body. Even with this correction the weights per unit of body weight of the liver, kidneys and heart are heavier in the oestradiol rats than in the castrated control animals (9·8, 2·0 and 0·88 g. respectively, as compared with 8·5, 1·5 and 0·75 g.). It must, however, be admitted that this correction is neither perfect nor complete, since the amount of fat present in the cells of the organs is not taken into consideration.

Macroscopically it was noticed that the livers of castrated control rats were light brown in colour, sharply differing from the dark, reddish-brown livers of the "oestradiol" rats. Preliminary histological investigation has shown that this difference in colour is due partly to hyperaemia in the livers of the oestradiol rats and partly to the much reduced amount of fat and lipoids in the liver cells of these animals as compared with those of castrated controls. These changes were present also in the livers of rats injected simultaneously with oestradiol and male hormones. No pathological changes were found in the livers after injections of male hormones alone, such livers appearing normal.

The changes in weight of the organs and the results of preliminary histological investigation of the liver and kidneys suggest that the male sex hormones most probably have a favourable stimulating effect upon the liver, kidneys, heart and spleen of castrated rats. At present the true nature of the activity of oestradiol on these organs is difficult to define until histological investigation is completed. The changes per unit of body weight, however, especially in the case of kidneys, may possibly indicate that oestrogens also have some stimulating effect upon these organs, veiled, however, by the stunted growth of the organism and fat impoverishment of the body.

Comparison of the effects on non-sexual organs with those on sexual organs

Two important points must be emphasized in the nature of the reaction of the different organs to castration and injections of the sex hormones:

(1) Castration and the injection of sex hormones into gonadectomized rats always cause typical changes in the sexual organs of every animal, while, in most cases, the weights of the non-sexual organs, fat deposition and gain in body weight are changed only in the majority of animals, the average figures, however, showing typical changes. If, however, the effects produced by the hormones are studied within the same litter, the rats of which are divided into the different groups, the number of animals showing the same typical changes is much increased, in several cases the exceptions being absent. The importance of comparison with control animals of the same litter has been emphasized in our previous papers [Korenchevsky, 1932; Korenchevsky *et al.* 1932; Korenchevsky & Dennison, 1934]. However, in the present experiments comprising 16 groups this was impossible, as our litters consisted of 4–8 rats only, which were divided into as many groups as possible.

(2) While prolongation of the injections of all hormones, except dehydroandrosterone, caused an increase in the effect upon the sexual organs, the effect upon the non-sexual organs was not increased, but even in some cases (e.g. testosterone) decreased. Probably this difference in reaction is at least partly to be explained by the changes produced in the gain in body weight and, perhaps, fat deposition, by the prolongation of the injections. As described above, the changes in the liver, kidneys, heart and spleen are much more dependent on the body weight than are those in the sexual organs.

Summary

1. The effects upon the sexual and some other organs of prolonged administration (about 3½ months) of sex hormones to castrated rats are described and compared with the results obtained in our previous experiments of shorter duration (about 23 days). The hormones injected were oestradiol dipropionate, androsterone, *trans*dehydroandrosterone, testosterone and testosterone propionate injected alone, or oestradiol dipropionate combined with the male hormones.

Effects on sexual organs

2. Prolongation of the injections was followed by a greater effect on the sexual organs in the case of androsterone, testosterone and testosterone propionate, but not of *trans*dehydroandrosterone.

3. Testosterone propionate was the only hormone which produced complete restoration of the atrophied sexual organs, or, with large doses, a great supernormal development of the seminal vesicles and prostate.

4. A comparatively small dose of oestradiol dipropionate (0·018 mg. per week) produced (for a female hormone) a great enlargement of the seminal vesicles, coagulating gland, prostate and even penis, but increasing the dose did not increase the effect.

5. As with other oestrogens, this apparent return towards normal weight and size of the sexual organs was of pathological nature and caused chiefly by increased development of fibrous tissue. This fibrosis should, however, be considered as one of the specific sex effects, as it was present only in the sexual organs.

6. With the doses used oestradiol dipropionate showed pronounced co-operation in restorative activity with testosterone, but not with testosterone propionate, while an antagonistic interaction with androsterone and *trans*-dehydroandrosterone (in contrast to the co-operative effect of androsterone with the weaker compound, oestrone) was found.

7. The neutralizing effect of the male hormones on the pathological changes in the sexual organs, produced by as strong an oestrogen as oestradiol dipropionate, was very pronounced in the case of testosterone and testosterone propionate, but weak with androsterone and *trans*dehydroandrosterone.

Body weight and fat deposition

8. Only oestradiol and, to a lesser degree, large doses of testosterone and testosterone propionate caused a decrease in the gain in body weight. Androsterone, *trans*dehydroandrosterone and small doses of testosterone and testosterone propionate, although having no effect when injected alone, partly neutralized the depressing effect upon growth of the simultaneously injected oestrogens.

9. All the hormones in the doses used caused in varying degree a decrease of the amount of body fat, except androsterone, which was even able in this respect to neutralize the effect of oestradiol.

Non-sexual organs

10. All the hormones accelerated the physiological involution of the thymus and there was a co-operative relation in this respect between the male hormones and oestradiol dipropionate.

11. The male hormones prevented to a considerable degree (with testosterone propionate in some cases almost completely) the tumour-like hyperplasia of the hypophysis produced by oestrogens.

12. The male hormones in most cases caused the "castration" adrenal to return to or towards normal, while oestradiol dipropionate produced specific pathological changes in this gland which were only partly neutralized by simultaneous injections of the male hormones.

13. Slight hypertrophy of liver, kidneys, heart and spleen was produced by the male hormones in most rats but oestradiol dipropionate caused either a decrease in their actual weights or (kidneys) no change, "dark" liver being a constant feature of all rats injected with this hormone.

14. This "oligosplanchnic" effect of oestrogens was to some extent neutralized by the male hormones, the comparatively weak sex hormone, androsterone, having the greatest effect in this respect.

Grants from the Medical Research Council, the Lister Institute and the Ella Sachs Plotz Foundation have enabled us to carry out this work and to them our thanks are due. We wish to express our gratitude to Messrs Ciba Ltd., in particular to Dr K. Miescher, for a supply of the hormones.

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XXVII. GLUTAMINE AND THE GROWTH OF *STREPTOCOCCUS HAEMOLYTICUS*

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(*Received 22 December 1938*)

In the course of our analysis of the nutrients of *Streptococcus haemolyticus* it was found that growth did not take place in a mixture containing peptone, cystine, glucose, phosphate and a number of other substances until an extract of meat also was added. Good growth then took place in some 16 hr. whereas in the culture without meat extract no growth was visible during the period of observation, usually 2 days but often 3 or 4 and on one occasion 9 days.

The test coccus was the well-known strain "Richards" maintained at high virulence by repeated passage through mice and kept in the intervals in rabbit blood. For the purposes of this work it was transferred from the blood to nutrient agar as required for the tests. After a variable number of subcultures on agar it was found that the coccus was of no further use as a test object in that it became able to grow on the peptone mixture without our extract of meat. This phenomenon of alteration in nutrient requirements we ascribed to the development of a power to synthesize our meat extract factor which was lacking while the organism was maintained in blood. Since the power to synthesize our factor was thus potentially present, positive results obtained by the addition of some fractions of meat, which were obtained only after 3 or 4 days' incubation, were suspect even though the control without the addition might be negative. In such cases it was possible that the fraction of meat did not contain the active substance we were seeking but did contain (*a*) a related compound from which our active substance was synthesized, or (*b*) some not necessarily specific substance which accelerated the enzymic processes utilized in the synthesis. In order therefore to ensure a test object which was incapable of growth in the absence of our meat extract we used a culture which was recovered from the blood every 7 days; and to satisfy ourselves that we were titrating our active substance itself and not something else which facilitated its synthesis we judged the potency of meat fractions by readings of growth up to 24 hr. and not 2 or 3 days.

Bacteriological technique

The general technique used on the bacteriological side of this work has followed that customary in this laboratory in nutritional studies of bacteria. The inoculum used has been relatively small. A small mass of bacteria was raked from an agar surface in such a way as to remove none of the medium and transferred to water. This was thoroughly suspended with a Pasteur pipette and diluted until the suspension was just invisible to the naked eye. One drop of this was used per tube. A precise enumeration of the number of cocci in a

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streptococcal suspension is impracticable but the number of colonies which grew on plating out the inoculum was usually about 1 million. It may, however, be stated that the strain used by us was capable of growing in the mixture to be described when the inoculum was reduced approximately to one coccus.

The inadequate basal medium used for the demonstration of the activity of the meat extracts was made as follows:

Bacto peptone (Difco)	10·0 g.
NaCl	5·0 g.
KH_2PO_4	4·5 g.
N NaOH	26 ml.
Water	to 700 ml.

Adjust to *p*H 7·4; distribute in 7 ml. lots in test tubes (6 in. × ¾ in.) and autoclave. At the time of testing add to each tube:

Sample to be tested (filtered)		1 ml.
M/18 phosphate buffer *p*H 7·4 (autoclaved)	1·05	2 ml.
M/20 cystine HCl in *N*/10 HCl (filtered)	0·2	
N/5 NaOH (autoclaved)	0·25	
Mixture *A*	0·5	
Inoculum	1 drop	

Mixture *A* consisted of:

Aneurin	0·005 g.	Thymine	0·01 g.
Nicotinamide	0·005 g.	Guanine	0·01 g.
β-Alanine	0·005 g.	KH_2PO_4	0·3 g.
Pimelic acid	0·05 g.	Glucose	5·0 g.
Uracil	0·01 g.	Riboflavin *M*/5000,	20 ml.
Cytosine	0·01 g.		

Uracil, cytosine, thymine and guanine were dissolved in 50 ml. *N*/10 H_2SO_4 and neutralized with NaOH to *p*H 6·0. The other ingredients were then added and water to 90 ml. Readjusted to *p*H 6·0 and made up to 100 ml. Filtered Seitz EK.

The tubes were incubated in an upright position at 37° in air + 5 % CO_2.

With regard to the constituents of mixture *A*, glucose is, of course, necessary as a source of energy but the part played by the others in growth has not yet been determined. In the absence of all, growth is usually but not always absent during the first day, but it may occur on the second day.

Chemical fractionation of meat

Extraction. The sodium sulphate method of Deutsch *et al.* [1938] was applied to fresh horse meat. The fat was cut from meat (20 lb.); the flesh minced (7·38 kg.; water content, 75 %), mixed with anhydrous Na_2SO_4 (2·77 kg.; ½ of the weight of water in the tissue) and the mixture twice reminced to a homogeneous stiff brown paste. This was heated with frequent stirring in a water bath at 45–50°; its temperature rose to 32° in the first 15 min. and remained so for a further 15 min. during the dissolving of the Na_2SO_4. The paste then became sloppier and its temperature rose. It was expressed at 35°; the extract cooled to room temperature; $Na_2SO_4, 10H_2O$ filtered off; the mother liquors cooled to 0° and more $Na_2SO_4, 10H_2O$ removed, leaving 1160 ml. of meat juice at about 2½ times its concentration in the tissues and containing about 4 % Na_2SO_4. In several

batches the yields ranged from 140 to 180 ml./kg. of fresh meat or about 50–60 % of the amount theoretically obtainable from the water content of the tissue. The efficacy of the various methods of separation attempted was judged by determination of the total N and growth activity of the fractions. Results obtained with the fractions described are given in Table III (p. 228).

First mercury precipitation. Mercuric acetate precipitated only part of the activity in the Na_2SO_4 extract; precipitation was complete under Neuberg's mercuric acetate-carbonate-alcohol conditions, but an equally complete precipitation was obtained with mercuric acetate in neutral 60 % alcoholic solution.

The extract (1000 ml.) was mechanically stirred and excess of finely powdered mercuric acetate (*c.* 100 g.) added, the solution neutralized with 10*N* NaOH (*c.* 60 ml.), alcohol (1500 ml.) added and the whole kept 2 hr. The precipitate was collected, freed from associated material by washing 3 times with water (500 ml.), stirred in water (500 ml.) in a closed jar and decomposed by H_2S under slight pressure. The HgS was filtered off and washed with water. The filtrate and washings from the sulphide contained almost the whole of the activity of the original extract and when evaporated in a vacuum gave a gum which retained its activity on keeping.

Phosphotungstic acid precipitation. Mercury-precipitated material equivalent to 1000 ml. of original extract was dissolved in 2*N* H_2SO_4 (300 ml.) and 20 % phosphotungstic acid in 2*N* H_2SO_4 added gradually with continuous stirring till in excess (*c.* 650 ml.). The precipitate was filtered after 4 hr. and washed 3 times with 2*N* H_2SO_4 containing a little phosphotungstic acid. H_2SO_4 and excess phosphotungstic acid were removed from the combined filtrate and washings, with baryta (*c.* 420 g.), excess Ba precipitated with H_2SO_4 and the solution filtered and evaporated under reduced pressure to 30 ml. Inorganic salts separated on keeping and were filtered off.

Properties of meat concentrates. The active material was not removed from aqueous solution by a wide variety of adsorbents and immiscible solvents at a variety of *p*H. In the Na_2SO_4 extract it was stable at 0° for several weeks. It was not very stable to heat: a little loss occurred at *p*H 5, 6 and 7 during heating for 1 hr. at 100° and $\frac{4}{5}$ of the activity was lost in 3 hr.; losses were slightly greater in all cases at *p*H 8 and 9. It was stable to mild oxidizing and reducing agents (air, silver salts; H_2S, SO_2). The following indications of its amino-acid nature were obtained: it was more completely precipitated by $Hg(CH_3CO_2)_2$-Na_2CO_3 in aqueous solution than by $Hg(CH_3CO_2)_2$ itself; it yielded a moderately insoluble copper salt which was not, however, useful in separation; it was destroyed by nitrous acid at room temperature in dilute aqueous solution and by alloxan during 10 min. at 100°.

Observations on glutamic acid and glutamine

At this point we observed the note by Rane & Subbarow [1938] in which they described the growth of *Str. haemolyticus* in a gelatin-hydrolysate medium containing, in addition to other substances, glutathione which they considered to be a "significant" constituent of the nutrients. We tested glutathione in the concentration suggested in combination with various constituents of our mixtures not referred to here and in particular as a replacement of our meat extract factor, and in the latter circumstances found that it gave a trace of activity, which trace was increased by increase in the concentration of glutathione. The activity of our meat extracts could not however be due to glutathione for the following reasons:

(1) Our active concentrates contained no demonstrable SS—SH group.

(2) Cuprous oxide, under the conditions described by Hopkins [1929] for glutathione, precipitated from our concentrates a small quantity of cuprous salts which contained only a trace of the original activity.

(3) Glutathione in concentrations equal in activity to our purer preparations, which were however still impure, contained about 5 times as much nitrogen.

(4) The stability and other properties of our material were different from those of glutathione.

The small activity of glutathione in growth tests would have suggested that this might be due to a biologically active impurity in our glutathione preparations, had not Rane & Subbarow found a synthetic preparation also active.

The somewhat trivial effects suggested that they might be non-specific in the sense already referred to and due merely to an improvement in the general metabolism brought about by increase in the amino-N content of the nutrients. In other work we had found that increases in the concentrations of particular amino-acids might have a critical effect upon growth. We therefore carried out tests in which the constituent amino-acids of glutathione, glycine and glutamic acid, were severally increased in concentration, cystine being already adequately represented.

Table I shows that high concentrations of synthetic glutamic acid were capable of inducing growth comparable in rapidity and mass with that resulting from our meat extract. It was shown that aspartic acid had no such action.

Table I. *Effect of glutamic acid as a replacement for meat extract*

A specimen of *dl*-glutamic acid synthesized by McIlwain & Richardson's [1939] method was dissolved in water, neutralized and autoclaved; concentration *M*/1·5. Dilutions were added to the inadequate mixture to give the concentrations shown.

	Growth after hr.		
	17	24	43
Inadequate medium + 0	0	0	0
+ *dl*-glutamate *M*/25	+ +	+ + + +	+ + + +
M/50	?	+	+ + + +
M/100	0	0	+ + + +
M/200	0	0	0
+ meat extract 1·0 ml.	+ + + +	+ + + +	+ + + +
0·2 ml.	+ + +	+ + +	+ + +
0·04 ml.	+	+	+ +
0·008 ml.	0	0	0

Here and elsewhere, *plus* signs are roughly proportional to the mass of growth.

Our interpretation of the results of Table I was that glutamic acid could not be the active substance in our meat preparations because there were obvious qualitative and quantitative differences between them. Glutamic acid might however be specifically associated with our active substance in the sense that the coccus could synthesize our active substance in adequate amounts in the presence of high concentrations of glutamic acid, but not in adequate amounts when the concentration of glutamic acid was low.

According to this interpretation glutamic acid might be the parent substance from which our active material was synthesized. We therefore surveyed the derivatives of glutamic acid which were known to occur in nature and which had similar properties to those of our active substance and we selected for test glutamine.

It was found that glutamine, sterilized by filtration, when added to the inadequate basal medium in a concentration as low as $M/5000$ caused full growth in as little as 16–17 hr. This growth was capable of serial subculture in the same mixture as often as desired. Asparagine tested under the same conditions was inactive.

The following specimens of glutamine from different sources had the same activity:

(1) A specimen isolated by Prof. Chibnall from natural sources, containing 98·2 % glutamine (glutamine was determined by the method of Vickery, Pucher Clark *et al.* [1935]).

(2) A specimen prepared by ourselves from beetroot by the method of Vickery, Pucher and Clark [1935], containing 97·5 % glutamine.

(3) A specimen synthesized by Prof. Harington from natural glutamic acid [Harington & Mead, 1935].

(4) A specimen synthesized by ourselves from synthetic glutamic acid.

The results with specimen 4 showed conclusively that glutamine itself and not any associated biologically active impurity was capable of replacing our active meat preparations.

A survey was then made of our various fractions of meat to estimate how far their activities could be ascribed to glutamine in them, determined as labile amide-N by the above method. The results of analysis are given in Table II on

Table II. *Glutamine content and activity of preparations*

	Total N mg./ml.	NH_3-N mg./ml.	Labile amide-N* mg./ml.	Calc. glutamine %	Activity for growth
Meat extract	27·7	0·37	0·106	0·111	+ + + +
1st Hg ppt.	4·59	0·36	0·105	0·109	+ + + +
Phosphotungstic filtrate	0·672	0·015	0·0403	0·042	+ + +

* Determined under the conditions of Vickery, Pucher, Clark *et al.* [1935] for glutamine.

solutions equivalent to the original meat extract. Negligible amounts of glutamine were found in the phosphotungstic precipitate and in the less soluble salts separated at that stage (p. 225); the loss of glutamine in the filtrate may readily be explained by the temporary alkalinity and the considerable evaporation involved in this process. It will be seen that the distribution of glutamine-N in the fractions was the same as that of growth activity.

Separation of glutamine from meat

The method used was essentially that by which Schulze & Bosshard [1883] and Vickery, Pucher & Clark [1935] isolated glutamine from plant extracts. It was not found possible to apply this to the immediate meat extract or first mercury precipitate owing to their containing much smaller amounts of glutamine than a typical beet extract (about 1/20) and larger amounts of associated materials. The method was however successfully applied to the material after phosphotungstic acid precipitation (p. 225) in which the glutamine, with 60 % loss, had been separated from 97·6 % of the associated nitrogenous impurities (Table II).

To material from 1000 ml. of meat extract at this stage, in water (50 ml.), saturated aqueous basic lead acetate solution was added till in excess (*c.* 40 ml.) while progressively neutralizing the solution with *N* acetic acid (*c.* 2 ml.). The pale cream-coloured granular precipitate settled quickly and was filtered off after 30 min. and washed 3 times with water (10 ml.). To the mother liquors

a 30 % solution of mercuric nitrate [cf. Vickery, Pucher & Clark, 1935] was added in excess (*c.* 30 ml.). The solution then had *p*H 2, and the white precipitate (Hg*P* I) was collected and washed after 2 hr. The mother liquors were neutralized with 10*N* NaOH (*c.* 6 ml.), kept overnight and the further precipitate (Hg*P* II) collected and washed with water. All fractions were decomposed with H_2S and their growth activities and glutamine contents determined. The material recovered from the lead precipitate had no activity and contained no detectable glutamine; the material from the two mercury precipitates contained the bulk of the glutamine and activity, while the mother liquors were slightly active and contained about 12 % of the total glutamine. The solutions containing the bulk of the material recovered from the two mercury precipitates were each neutralized with ammonia, evaporated under reduced pressure from a bath at 50° to about 2 ml. and the separated crystals collected and washed with 50 % aqueous alcohol. More crystals were obtained from their mother liquors by treatment with alcohol. The fractions had the properties shown in Table III. The activities of these fractions also were closely correlated with their glutamine contents, and about ½ the glutamine in the starting material had been isolated.

Table III. *Characters and growth activities of glutamine fractions isolated from meat*

	Wt. g.	M.P.	Mixed M.P. with *l*-(+) glutamine	Glutamine content %	Glutamine content g.	Activity*
Hg*PI* 1st crop	0·125	194–5°	196–7°	99	0·22	+ + +
Hg*PI* 2nd crop	0·036	194–5°	196–7°			
Hg*PI* 3rd crop	0·087	172–4°	187–9°	69		
Hg*PI* mother liq.					0·013	+
Hg*PII* 1st crop	0·076	>260°		0	0	0
Hg*PII* 2nd crop	0·027	>260°				
Hg*PII* mother liq.					0·120	+ +
Initial phosphotungstic acid precipitated meat extract					0·420	+ + + +

* Tested in concentration equivalent to that of the original meat extract.

Table IV. *The growth activity of meat in relation to its glutamine contact*

Crude meat extract estimated to contain 0·111 mg. glutamine/ml. was titrated against a solution of glutamine (specimen 3) containing 0·111 mg./ml.

Tube			Growth in hr. 24	29½	48½
1	Inadequate medium + glutamine	1·0 ml.	+ + + +	+ + + +	+ + + +
2	+ glutamine	0·2 ml.	+ + +	+ + + +	+ + + +
3	+ glutamine	0·04 ml.	+ +	+ + ⊥	+ + +
4	+ glutamine	0·008 ml.	Tr.	+	+ ⊥
5	+ glutamine	0·0016 ml.	0	Tr.	+
6	+ glutamine	0·00032 ml.	0	0	0
7	+ meat extract	1·0 ml.	+ + + +	+ + + +	+ + + +
8	+ meat extract	0·2 ml.	+ + +	+ + + +	+ + + +
9	+ meat extract	0·04 ml.	+ +	+ + ⊥	+ + +
10	+ meat extract	0·008 ml.	Tr.	+	+ ⊥
11	+ meat extract	0·0016 ml.	0	0	0
12	+ meat extract	0·00032 ml.	0	0	0
13	+ water	1·0 ml.	0	0	0

The purer glutamine fractions were recrystallized 3 times from aqueous alcohol; the product melted higher (205°) than glutamine previously isolated from natural sources, had a typical optical rotation of $[\alpha]_D^{21°} = +6{\cdot}5 \pm 0{\cdot}1°$ and gave the precipitation reactions described in the literature.

In a final experiment it was shown that under the conditions of our test the only contribution made by the meat to the growth of streptococcus was glutamine. A solution of glutamine was prepared at a concentration equivalent to the estimated concentration in our crude meat extract and both were titrated for growth-inducing activity. Table IV shows that the activity of each was approximately the same.

Discussion

The use of meat extract as an addition to peptone was, according to Bulloch [1930], introduced by Loeffler in 1881 and has been a routine procedure in medical bacteriology ever since. It would appear that an important function of the meat so far as *Str. haemolyticus* is concerned is to ensure the presence of glutamine in the nutrients. Arguing from the action of animal extracts and fluids (e.g. urine) upon the growth of streptococcus it may be assumed that glutamine is widely distributed in the animal body, but little reference can be found to its possible function. It is however interesting to note that Krebs [1935] has found that the brain cortex and retina of vertebrates and the kidney of the rabbit and guinea-pig can synthesize glutamine from glutamic acid and that extracts of these organs contain a glutaminase which can catalyse the reverse process. It is possible that similar processes can be carried out by streptococci and that they are a necessary feature of their growth.

The effect of glutamine described has also been demonstrated with ten other strains of haemolytic streptococci. Two others grew equally well under the conditions used without glutamine.

Summary

This work continues a study of the nutrients essential for the growth of bacteria. Previous work has shown that "simple" non-pathogenic microbes can obtain all nitrogenous compounds required in their metabolism by synthesis from ammonia. Increasing complexity of nutrient requirements, usually associated with increasing pathogenicity, calls for the provision of more complex nutrients some of which have been identified with established animal vitamins. The present paper shows that glutamine, a substance present in animal tissues but with no known function, is an "essential growth factor" for most strains of streptococci isolated from cases of disease in that it must be supplied in the nutrients.

We have pleasure in thanking Prof. A. C. Chibnall and Prof. C. R. Harington for supplying us with specimens of glutamine and Dr L. Colebrook and Dr L. E. H. Whitby for cultures used in the tests.

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XXVIII. THE RETENTION OF ACIDS BY SERUM BASED ON THE RETENTION COEFFICIENT: ITS USE IN MANOMETRIC EXPERIMENTS

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(*Received 30 December 1938*)

THE two-vessel method of Warburg [1924] has the unique advantage for the measurement of tissue metabolism that it permits the simultaneous and continuous reading of respiration and total acid production. Whilst serum may be used as the medium for such measurements [Warburg, 1925], and is a more physiological medium than simple saline solutions, the retention of part of the evolved lactic or carbonic acid caused by its interaction with the buffers other than bicarbonate brings with it the need for special retention corrections.

Hitherto, two procedures have been available: (1) In the original method of Warburg [1925] the retentions for lactic and carbonic acids are calculated from the data of a separate acidification experiment, and from the bicarbonate and CO_2 contents of the serum. The correction obtained applies only to experiments in which the change of CO_2 pressure is close to that found in the acidification experiment. (2) Some of the inconvenience of the former method is avoided by acidification in the actual vessels used for measurement of tissue metabolism [Warburg *et al.* 1931], all of which must therefore be provided with side-bulbs. This is not always convenient, and some loss of accuracy may result from the fact that the acidification is made with the tissue present in the vessels. With some tissues the addition of lactic acid may cause an appreciable change in metabolism. Even slight errors in reading may cause an appreciable error in the retention determination, and it is not easy to secure high accuracy in presence of respiring or glycolysing tissue. In this method also, the retention correction applies only to a pressure change close to that found in the acidification experiment. Separate retention estimations for CO_2 and lactic acid are necessary.

In the principle to be described, instead of basing the calculation of retention on the Michaelis dissociation residue, as was done by Warburg [1925], we have worked from the empirical finding that the retention, or decrease of alkali proteinate, is directly proportional to the change of *p*H. This was first shown by Van Slyke *et al.* [1923] for dialysed serum, and afterwards by Dickens & Šimer [1932] and Dixon [1937] for normal serum. Over the physiological range of *p*H the relationship may be regarded as linear, and an essential simplification is thus introduced. It is also possible with this method to calculate the retention for any observed pressure change.

Nomenclature

The following symbols are used throughout:

H^+ = hydrogen ion concentration of the serum.

K = 1st dissociation constant of carbonic acid (for serum we take $K = 7{\cdot}94 \times 10^{-7}$, $pK = 6{\cdot}10$ [Hastings *et al.* 1928]).

p = partial pressure of CO_2 (mm. Brodie solution).

B = bicarbonate concentration (μl. per ml.).

β = solubility coefficient of CO_2 in serum (μl. CO_2/ml. for a pressure of 1 atm. see below).
v_F = volume of serum in vessel (ml.).
Δu = retention of CO_2 per ml. serum per mm. change of p (where u is the hypothetical concentration of the acidic buffers other than bicarbonate, e.g. proteinates, in the serum, expressed in μl./ml. as usual).

All pressures are reckoned in mm. Brodie fluid, of which 10,000 mm. = 1 atm. Volumes of gas, or of acids and bases considered as gases in the usual convention, are in μl. at N.T.P. Volumes of liquids are in ml. throughout.

With these units, the solubility coefficient, β, is equal to $\alpha/10$, where α is the Bunsen absorption coefficient of the gas. For values of α for various salt solutions and for serum see Van Slyke *et al.* [1928].

The "Retention Coefficient" R

Since within the limits shown by Dickens & Šimer [1932] the retention-pH curve of normal serum is linear, we may write

$$-\frac{du}{d\,(p\mathrm{H})}=R \qquad \text{.....(1),}$$

where R is an empirical constant which will be called the retention coefficient, denoting the retention by 1 ml. serum, expressed in μl. per unit change of pH. R is a positive quantity, and its value varies between about 100 and 200 for normal sera of different species.

Now
$$R=-\frac{du}{d\,(p\mathrm{H})}=-\frac{du}{d\mathrm{H}^+}\,\frac{d\mathrm{H}^+}{d\,(-\log_{10}\mathrm{H}^+)}=2{\cdot}303\,\frac{du}{d\mathrm{H}^+}\,\mathrm{H}^+ \qquad \text{.....(2).}$$

It may be shown (Table I) that, to a first approximation, taking finite increases and the mean value of H^+ over the interval H_0^+ to $(H_0^+ + \Delta H^+)$

$$R=2{\cdot}303\,\frac{\Delta u}{\Delta \mathrm{H}^+}\left(\mathrm{H_0}^+ + \frac{\Delta \mathrm{H}^+}{2}\right) \qquad \text{.....(2}a\text{),}$$

whence
$$\frac{\Delta \mathrm{H}^+}{\mathrm{H_0}^+}=\frac{2{\cdot}303\Delta u}{R-\dfrac{2{\cdot}303\,\Delta u}{2}} \qquad \text{.....(3).}$$

Table I. *Accuracy of equation (3)*

From equation (1) $\Delta u/R = -\Delta p\mathrm{H}$. From equation (2$a$) $\Delta u/R \sim \Delta \mathrm{H}^+/2{\cdot}303\left(\mathrm{H_0}^+ + \dfrac{\Delta \mathrm{H}^+}{2}\right)$. In Table I these two values of $\Delta u/R$ are compared. The difference is the error in equation 2a.

			$\Delta u/R$		
			$\dfrac{\Delta H^+}{2{\cdot}3\left(\mathrm{H_0}^+ + \frac{\Delta \mathrm{H}^+}{2}\right)}$		
pH	$H^+ \times 10^8$	$\Delta H \times 10^8$		$-\Delta p$H	Difference %
7·3979	4 (= H_0)	—	—	—	—
7·3468	4·5	0·5	0·0510	0·0511	0·2
7·3010	5	1	0·0965	0·0969	0·4
7·2596	5·5	1·5	0·1369	0·1383	1·0
7·2218	6	2	0·1737	0·1761	1·4

Acidification of serum

1. *General case.* The partial pressure of CO_2 in equilibrium with the serum increases from p_0 to $p_0+\Delta p$. Since

$$p_0=B_0\mathrm{H_0}^+/K\beta \quad \text{and} \quad p_0+\Delta p=(B_0+\Delta B)\,(\mathrm{H_0}^+ + \Delta \mathrm{H}^+)/K\beta$$

therefore

$$\frac{\Delta p}{p_0}-\frac{\Delta B}{B_0}=\frac{\Delta \mathrm{H}^+}{\mathrm{H_0}^+}\left(1+\frac{\Delta B}{B_0}\right) \qquad \text{.....(4).}$$

Substituting from equation (3) the value of $\Delta H^+/H_0^+$

$$\frac{\Delta u}{\Delta p}=\frac{R\left(1-\frac{p_0\Delta B}{B_0\Delta p}\right)}{2{\cdot}303\ p_0\left(1+\frac{\Delta B}{2B_0}+\frac{\Delta p}{2p_0}\right)} \qquad \text{.....(5).}$$

Equation (5) represents the general case when p, B and u vary. Two special cases are important.

(*a*) *Acidification of serum by addition of lactic or other strong acid.* In a vessel of total volume v ml., containing v_F ml. serum and v_T ml. total liquid including serum, maintained at a temperature of $T°$ absolute, let m μl. lactic acid be added to the serum, and the resulting increase of partial pressure of CO_2 be Δp mm. Brodie.

The "vessel constant for Ringer solution" $k^R_{CO_2}$ [Warburg, 1925] is given by

$$k^R_{CO_2}=\frac{273}{10T}(v-v_T)+v_T\beta.$$

Change of bicarbonate concentration on acidification $=\Delta B$

$$\Delta B=-\Delta p\ k^R_{CO_2}/v_F.$$

Substituting in (5) and writing the quotient with suffix L, denoting acidification by lactic acid

$$\left(\frac{\Delta u}{\Delta p}\right)_L=\frac{R}{2{\cdot}303}\frac{1+\frac{p_0\,k^R_{CO_2}}{B_0 v_F}}{p_0+\frac{\Delta p}{2}\left(1-\frac{p_0\,k^R_{CO_2}}{B_0\,v_F}\right)} \qquad \text{.....(6).}$$

Equation (6) shows that the retention for lactic acid is dependent on the vessel constant and volume of serum. In these respects it differs from the retention for CO_2. The term in Δp indicates the extent of variation of the retention with CO_2 pressure-change, which was not previously calculable.

(*b*) *Acidification of serum by addition of carbonic acid.* Since for each equiv. of CO_2 which is combined, 1 equiv. of HCO_3^- results, accompanied by an increase of 1 equiv. in u, in this case

$$\Delta u=\Delta B.$$

Substituting in (5), and writing the quotient with suffix C, denoting retention for CO_2

$$\left(\frac{\Delta u}{\Delta p}\right)_C=\frac{R}{2{\cdot}303\left\{p_0+\frac{\Delta p}{2}\left(1+\frac{p_0\,\Delta u}{B_0\,\Delta p}\right)\right\}+\frac{Rp_0}{B_0}} \qquad \text{.....(7).}$$

Equation (7) shows that the retention for CO_2 is independent of the vessel constant and the volume of serum, but depends on the particular value of Δp as is evident from the bracketed term in the denominator. This term, involving $(\Delta u/\Delta p)$ is nearly equal to $p_0+\frac{\Delta p}{2}$ unless p_0 is large compared with B_0, since the average value of $(\Delta u/\Delta p)$ is of the order 0·1. Consequently we may first calculate an approximate value of $(\Delta u/\Delta p)$ using the approximate relationship

$$\left(\frac{\Delta u}{\Delta p}\right)_C\sim\frac{R}{2{\cdot}303\left(p_0+\frac{\Delta p}{2}\right)+\frac{Rp_0}{B_0}} \qquad \text{.....(7}a\text{),}$$

and afterwards insert this value of $(\Delta u/\Delta p)$ in the denominator of equation (7). In many cases the effect of this correction term will be almost negligible, but the calculation is very easy in practice, so that it is better to insert the correction as a routine.

Determination of the retention coefficient, R

Since R represents the retention per unit change of pH, it may be determined by any of the three methods available for determining retention [Warburg, 1925]. These are (1) acidification with a known amount of lactic acid: (2) acidification with a known amount of CO_2 and (3) dilution of the serum. The third method is complicated by the fact that since R is dependent on the concentration of non-bicarbonate buffers, the value of R will change during dilution. For this reason methods (1) and (2) are preferable.

The calculation of R may also be made by either of two methods: (*a*) R may be derived from the above equations (6) and (7) for $(\Delta u/\Delta p)$ or (*b*) R may be calculated directly from the data of the acidification experiment, using a logarithmic method. Since these calculations afford an excellent check on the accuracy of the various expressions, they will be described separately.

1*a*. *Calculation of* R *by acidification with lactic acid: derivation from* $(\Delta u/\Delta p)_L$. Let ϵ ml. of a solution of lactic acid containing m μl. acid be added to v_F ml. serum contained in a vessel of Ringer constant $k^R_{CO_2}$, and let the observed pressure change be h mm. Brodie. Let the pressure change due to dilution of the serum thus caused be h' mm.; it should be noted that h' is negative [Warburg, 1925] and its sign must be taken algebraically. Let H be the pressure change due to acidification alone, without dilution.

Then
$$H = h - h',$$
$$\Delta u = \frac{m - Hk^R_{CO_2}}{v_F},$$
$$\Delta p = h.$$

Hence $(\Delta u/\Delta p)_L$ is directly calculated from the acidification experiment. If now ϵ is a small volume, e.g. $< 5\,\%$ of v_F, R will remain sensibly constant during dilution. Hence from equation (6)

$$R = \frac{2{\cdot}303\,(m - Hk^R_{CO_2})\left(p_0 + \frac{h}{2} - \frac{h\,p_0\,k^R_{CO_2}}{2v_F B_0}\right)}{h\left(v_F + \frac{p_0\,k^R_{CO_2}}{B_0}\right)} \qquad \text{.....(8).}$$

1*b*. *Calculation of* R *from acidification with lactic acid: logarithmic method.* In the above experiment, the initial conc. of bicarbonate $= B_0$. Final conc. of bicarbonate after acidification

$$= B_1 = \frac{B_0 v_F - hk^R_{CO_2}}{v_F + \epsilon}.$$

Hence
$$\Delta p\mathrm{H} = p\mathrm{H}_1 - p\mathrm{H}_0 = \log\frac{B_1}{(p_0 + h)\,\beta} - \log\frac{B_0}{p_0\beta}$$

and
$$\Delta u = (m - hk^R_{CO_2})/v_F.$$

Therefore
$$R = -\frac{\Delta u}{\Delta p\mathrm{H}} = +\frac{m - hk^R_{CO_2}}{v_F} \Bigg/ \log\frac{\left(1 + \frac{\epsilon}{v_F}\right)\left(1 + \frac{h}{p_0}\right)}{1 - \frac{hk^R_{CO_2}}{B_0 v_F}} \qquad \text{.....(9).}$$

This equation eliminates the need for a dilution correction, and is the best form to use.

2*a*. *Calculation of* R *from acidification with* CO_2*: logarithmic method.* In this case the acidification occurs in a special vessel of the type described by Warburg *et al.* [1931]. An outer compartment contains $NaHCO_3$-NaCl solution, into which m μl. lactic acid are tipped, thus liberating m μl. CO_2. In an inner compartment of the same vessel are v_F ml. serum. Part of the CO_2 liberated from the

$NaHCO_3$ recombines with the buffers of the serum. The pressure change observed is h mm.

Then
$$\Delta u = (m - hk^R_{CO_2})/v_F,$$
$$\Delta p\mathrm{H} = \log\left(1 + \frac{m - hk^R_{CO_2}}{B_0\, v_F}\right)\left(\frac{p_0}{p_0 + h}\right) \qquad \text{.....(10).}$$

Whence $R = -(\Delta u/\Delta p\mathrm{H})$ is readily calculated.

2*b*. *Calculation from equation* (7). In the above experiment, Δu is given and $h = \Delta p$. Hence the quotient $(\Delta u/\Delta p)_C$ is directly obtained and from (7):
$$R = \frac{2{\cdot}3\,(\Delta u/\Delta p)}{1 - \frac{p_0}{B_0}\frac{\Delta u}{\Delta p}}\left\{p_0 + \frac{\Delta p}{2}\left(1 + \frac{p_0}{B_0}\frac{\Delta u}{\Delta p}\right)\right\} \qquad \text{.....(11).}$$

Vessel constants

When the values of $(\Delta u/\Delta p)_C$ and $(\Delta u/\Delta p)_L$ for the required range of Δp have been calculated by means of equations (6) and (7), it is only necessary to insert these values in equations (12) and (13) to obtain the respective vessel constants for serum [Warburg, 1925].
$$k^S_{CO_2} = k^R_{CO_2} + v_F\left(\frac{\Delta u}{\Delta p}\right)_C \qquad \text{.....(12),}$$
and
$$k^S_L = k^R_{CO_2} + v_F\left(\frac{\Delta u}{\Delta p}\right)_L \qquad \text{.....(13).}$$

The calculation of the result of a 2-vessel metabolic experiment may then be made either by the original Warburg [1925] method, or by the method of Warburg *et al.* [1931]. Average values of R.Q. for various tissues, which are required for the latter method, are given by Dickens & Šimer [1930; 1931, 2].

Retention in the Dickens-Šimer R.Q. method

In the direct method for measurement of metabolism in bicarbonate media [Dickens & Šimer, 1931; 1932; Dixon & Keilin, 1933; Dixon, 1937] the measurement of respiration is not affected by retention, unlike the same measurement by the Warburg 2-vessel method. But for the measurement of glycolysis by these methods the present principle has the advantage of greater accuracy compared with the "retention curve" estimations previously described [Dickens & Šimer, 1932; Brekke & Dixon, 1937]. $\Delta p\mathrm{H}$ being known directly from the manometric readings [cf. Dickens & Šimer, 1932; 1933] it is only necessary to multiply this by R in order to obtain the actual retention, in μl. per ml. serum, which occurred during the experiment. R is determined as usual by acidification by lactic acid. Only two extra vessels (with Brodie fluid in the manometers) are needed, one for acidification of serum and one for estimation of the strength of the lactic acid (cf. Table IV, vessels 2 and 4).

Dilution correction

When R is determined from the value of $(\Delta u/\Delta p)_L$ (but not by the logarithmic method) a correction h' due to the change of $p\mathrm{H}$ of the serum on dilution with the small volume ϵ has to be applied (equation 8). The separate determination of h' is generally unnecessary, since it may be readily calculated from the value of R, as follows (the suffix D denotes dilution):
$$\Delta \mathrm{H}^+_D = \frac{\beta K\,(p_0 + h')}{B_0\frac{v_F}{v_F + c} - \frac{h'\,k^R_{CO_2}}{v_F + \epsilon}} - \frac{\beta K\, p_0}{B_0} \qquad \text{.....(14).}$$

If h' is small (it is *c.* -5 mm. for $\epsilon = 0{\cdot}1$ ml.) and B_0 and $k^R_{CO_2}$ are of the usual order (*c.* 500 and 1 respectively), $h'k^R_{CO_2}$ is negligible in relation to $B_0 v_F$ and since p_0 is usually *c.* 400, $(p_0 + h') \sim p_0$

$$\Delta H_D{}^+ = \frac{\beta K p_0}{B_0}\left(\frac{\epsilon}{v_F}\right) = H_0{}^+ \frac{\epsilon}{v_F}$$

and

$$\Delta u = -\frac{h k^R_{CO_2}}{v_F}$$

Hence from equation (2), regarding R as constant for this slight dilution,

$$h' = -\frac{R\epsilon}{2{\cdot}3\, k^R_{CO_2}} \qquad \text{.....(15).}$$

For the purpose of equation (15), R may be given an approximate value of 150, provided that the volume ϵ does not exceed 0·1 ml. The error in h' is then unlikely to exceed *c.* 1·5 mm. in an extreme case. If greater accuracy is desired the logarithmic method of calculation (equation 9) may be used. The dilution effect may be avoided completely by using evaporated tartaric acid solution instead of lactic acid [Warburg, 1925] and writing $\epsilon = 0$ and $h' = 0$ in the above equations (8) and (9), but the use of a solution is convenient.

Typical case

For an average specimen of normal serum at 37·5° the following values are typical: $R = 150$, $p_0 = 420$, $B_0 = 427$, $\alpha = 0{\cdot}51$ [Van Slyke *et al.* 1928], or $\beta = 0{\cdot}051$, $p_0\beta = 21{\cdot}4$, $pK = 6{\cdot}10$ or $K = 7{\cdot}94 \times 10^{-7}$ [Hastings *et al.* 1928], $pH_0 = 7{\cdot}40$ or $H_0{}^+ = 4 \times 10^{-8}$.

Table II shows values of $\Delta u/\Delta p$ for this specimen of serum. The values of $(\Delta u/\Delta p)_C$ are calculated from equation (7). Correction of the value (e.g. for $\Delta p = 100$) by means of equation (7*a*) only changes the value from 0·122 (uncorr.)

Table II. *Calculation of* $(\Delta u/\Delta p)$ *and* ΔpH *for a typical specimen of serum*

For details see text. Lactic retention calc. for a vessel of total volume 13·2 ml.; for $v_F = 2$ ml., $k^R_{CO_2} = 1{\cdot}09$; for $v_F = 7$ml., $k^R_{CO_2} = 0{\cdot}902$. $R = 150$.

Retention for CO_2

Δp mm. Brodie	$(\Delta u/\Delta p)_C$ from equation (7)	$\Delta u = \Delta p(\Delta u/\Delta p)_C$	$\Delta pH = -\Delta u/R$
0	0·135	0	0
50	0·126	6·3	−0·042
100	0·119	11·9	−0·079
150	0·112	16·8	−0·112
200	0·106	21·2	−0·141

Retention for lactic acid; $(\Delta u/\Delta p)_L$ calc. from equation (6)

Δp mm. Brodie	For $v_F = 2$			For $v_F = 7$		
	$(\Delta u/\Delta p)_L$	Δu	ΔpH	$(\Delta u/\Delta p)_L$	Δu	ΔpH
0	0·240	0	0	0·175	0	0
50	0·234	11·7	−0·078	0·166	8·3	−0·055
100	0·228	22·8	−0·152	0·158	15·8	−0·105
150	0·222	33·3	−0·222	0·151	22·6	−0·151
200	0·216	43·2	−0·288	0·145	29·0	−0·193

to 0·119 (corr.), an amount which is almost negligible in practice. On the other hand the effect of the correction for various values of Δp is quite large, and this explains why with the Warburg [1925] method of calculation, the retention could only be determined for one particular experimental value of Δp.

The values of $(\Delta u/\Delta p)_L$ in Table II are calculated from equation (6) for the same specimen of serum. For the same vessel, two series of values are given to show the effect of varying v_F. The effect of Δp resembles that found for retention of CO_2 (Table II).

For a vessel of $k^R_{CO_2}$ about 1·0, an increase of p of about 100 mm. Brodie causes a decrease of the vessel constant for serum of about 5%. The effect of this in an experiment by the 2-vessel method may be to alter the metabolic quotient by an amount which may reach 10% of its value.

The change in pH corresponding to any value of Δp may be readily computed from the equation: $\Delta p\mathrm{H} = -\Delta p\,(\Delta u/\Delta p)/R$, in which it is important to use the correct quotient and value of Δp for CO_2 or lactic acid respectively. The values of ΔpH included in Table II show the order to be expected. This calculation is useful for two purposes; it enables one to guard against too great an acidity of the medium, and it may also be used in conjunction with Table I as a check on the error introduced by the approximation in equation (2*a*).

Practical details

Serum. The serum used in these experiments was obtained by allowing the blood to clot, centrifuging the supernatant serum, and inactivating by heating for two successive periods of 2 hr. at 56°.

The serum was warmed to 37° and shaken with the gas-mixture before use. It is advisable, and with larger volumes of serum essential, to pass the gas stream through the vessels containing the serum, while they are shaking in the bath at 37°. A comparison of the retention of 7 ml. serum contained in a vessel of total vol. 14 ml. gave, by previous gassing in the laboratory and then transferring to the bath $(\Delta u/\Delta p)_M = 0{\cdot}131$; by gassing while shaking in the bath $(\Delta u/\Delta p)_M = 0{\cdot}193$. Consequently the vessels should be provided with bored-out stoppers, and the gassing arrangement recommended by Dickens & Šimer [1933, p. 450] should be used.

Lactic acid solution. The solution, *c.* $M/20$, is refluxed for about 1 hr., cooled, NaCl added to make the conc. 0·9% and the strength of the solution determined manometrically. The strength remains unchanged for many weeks if the solution is kept sterile by occasionally heating in the stoppered flask in a boiling water bath.

Accurate measurement of the solution into the side-bulbs by a good, grease-free micro-pipette is essential.

The details of a complete retention estimation are given in Table III. The gas analysis is by the method described by Dickens & Šimer [1931, 1], using Clerici solution of density *c.* 4 as manometric fluid. If a Brodie thermobarometer is used, its readings must be divided by 4 before correcting the pressures in the gas analysis. Naturally the gas analysis is needed only when a new cylinder of gas is employed; correction of p_0 for various temperatures and barometric pressures may easily be made, if necessary.

In general, the acidification by lactic acid is to be preferred to the direct determination of $(\Delta u/\Delta p)_C$ as in vessel 1, Table III. Owing to the larger vessel volume necessary, the latter method is less accurate and cannot easily be applied to larger volumes of serum. An example is shown in Table III, for which the bicarbonate-R.Q. vessel of Dickens & Greville [1933] was used, and proved quite suitable; but it will be seen that the value of Δu obtained is greatly affected by a very small error in h. Consequently it is better to calculate $(\Delta u/\Delta p)_C$ from an experiment of the type shown in vessel 2. If the volume of serum is limited, 2 or 3 ml. may be used, or less with smaller vessels. The latter

Table III. *Protocol of a complete retention estimation*

Pig serum. 37·5°. O_2+CO_2 mixture passed through all vessels for 8 min. while shaking in the bath. α_{CO_2} for serum = 0·51, for Ringer solution = 0·54. Manometer fluid: Brodie solution, except No. 5 which was Clerici fluid of density 3·717. Strength of lactic acid solution: *c.* *N*/20 in 0·9% NaCl. Vessels: No. 1 bicarbonate-R.Q. type [Dickens & Greville, 1933]; Nos. 2 and 4 rectangular with side-bulb; Nos. 3 and 5 conical with side-bulb. All vessels with bored-out stoppers for gassing in bath. Barometer 758·2 mm. Hg.

Vessel no. ...	1	2	3	4	5
Exp.	CO_2 retention	Lactic retention	Bicarbonate estimation	Strength of lactic acid	Gas analysis
Vessel vol. (ml.)	23·72	13·52	16·36	14·56	*c.* 20
v_T	4·10	7·10	0·70	2·1	2·3
v_F (serum)	2	7	0·5	—	—
$k^R_{CO_2}$	1·943	0·926	1·411	1·206	—
Vessel contents:					
Main pt.	2 ml. serum	7 ml. serum	0·5 ml. serum	2 ml. $NaHCO_3$-Ringer	2 ml. *M*/5 $KMnO_4$ in *N*/500 H_2SO_4
Outer ring	2 ml. $NaHCO_3$-Ringer	—	—	—	—
Side-bulb	0·1 ml. lactic acid	0·1 ml. lactic acid	0·2 ml. *N* HCl	0·1 ml. lactic acid	0·3 ml. 30% NaI, $2H_2O$ in *N*/500 H_2SO_4
Pressure change on mixing (corrected for thermobarometer vessel):					
h	+49·5	+46·5	+155·5	+92 (Brodie)	* −81 (Clerici)
	$hk^R_{CO_2}+96$	+43	$2hk^R_{CO_2}=B_0=439$	$hk^R_{CO_2}=m=111$	$p_0=81\times\frac{3\cdot717}{13\cdot60}\times\frac{10,000}{760}$ =290 mm. Brodie
Dilution corr.	$h'=0$	$h'=-5$	—	—	—
$(h-h')\,k^R_{CO_2}$	+96	+48	—	—	—
$(m-Hk^R_{CO_2})/v_F=\Delta u$:	7·5	9·0	—	—	—
$\Delta u/h$	$(\Delta u/\Delta p)_C=0·151$ for $\Delta p\sim 50$	$(\Delta u/\Delta p)_L=0·193$ for $\Delta p\sim 45$	—	—	—
Calculation according to Warburg [1925]:	$(\Delta u/\Delta p)_C=0·153$ for $\Delta p_C=59$	$(\Delta u/\Delta p)_L=0·192$ for $\Delta p_L=46$	—	—	—
Calculation of R:					
(*a*) From equations (8) and (11)	$R=123$	$R=127$	—	—	—
(*b*) From equations (9) and (10)	$R=123$	$R=127$	—	—	—
Calculation of $(\Delta u/\Delta p)$, for mean value of $R=127$, from equations (6) and (7), for above values of Δp:	$(\Delta u/\Delta p)_C=0·152$	$(\Delta u/\Delta p)_L=0·193$	—	—	—

* Corrected for pressure change of −3 mm. on mixing reagents [Dickens & Šimer, 1931, 1].

must be carefully made to provide a large surface of the serum, or equilibration with the gas mixture is slow. When using small volumes of serum care must be taken that the dilution effect is small (equation 15), and it may therefore be necessary to use evaporated tartaric acid instead of lactic acid.

Since the gas analysis will generally be done at once when the gas mixture is received, only three vessels are necessary for a complete retention estimation, viz. nos. 2, 3 and 4, Table IV. From the values of h, B_0 and m thus obtained, the retentions for lactic and carbonic acids for any desired values of Δp may at once be calculated by means of equations (9), (6) and (7). The value of R obtained

from equation (9) may be used to calculate the retention in any experiment in which the same specimen of serum is used at a value of pH within the physiological range.

Relation of retention to protein content of serum

According to Van Slyke *et al.* [1923] for dialysed horse serum the empirical relationship

$$\frac{BP}{(p\text{H}-4{\cdot}80)}=0{\cdot}68P$$

holds for the physiological range, where BP is the millimolar conc. of base bound by protein, and P the total protein in g./100 ml. Hence in our units for u

$$\Delta u=-\Delta\,(BP)\times 22{\cdot}4,$$

and

$$R=-du/d\,(p\text{H})=0{\cdot}68\times 22{\cdot}4\,P=15{\cdot}2\,P.$$

Table IV shows the relationship of R to P for 6 specimens of (undialysed) normal serum. The variation of R from $15P$ to $22P$ is rather large, not only with different species, but also with different sera of the same species. Hence it is not practicable to calculate R from the protein content of the serum, but is better to determine it manometrically for each specimen. The coincidence of the retention-pH curves for two particular specimens of horse-serum [Dickens & Šimer, 1932] and sheep-serum [Dixon, 1937] must therefore be regarded as fortuitous.

Table IV. *Value of* R *and total protein in various specimens of serum*

Species ...	Pig	Calf	Sheep I	Sheep II	Horse I	Horse II
Mean R*	131	108	183	108	106	107
Total protein %	7·20	4·98	6·25	6·42	7·10	6·35
R/(% protein)	18·2	21·6	22·2	16·8	15·0	16·9

* Mean of 7 estimations.

Retention by substances of known pK′

In manometric experiments made in simple $NaHCO_3$-saline media, it is often necessary to consider if the retention of added substrates or other substances need be taken into account. Table V shows the retentions due to substances of various pK, for different reactions of the medium.

Table V. *Value of* R *for various values of* K′

For a substance of dissociation constant K', dissolved in conc. c' (μl./ml.) in a bicarbonate medium in equilibrium with CO_2 [Dickens & Šimer, 1932]

$$R=-\frac{d\rho}{d\,(p\text{H})}\,c'=2{\cdot}3\,\frac{\text{H}^+K'}{(\text{H}^++K')^2}\,c'.$$

The following Table shows values of R for various values of H^+ and K': the value of c' taken is equivalent to $M/130$ and gives $R_{max}=100$.

	R for $c'=174$ at		
pK'	pH = 6·0	pH = 7·0	pH = 8·0
4	4	0·4	0·04
5	33	4	0·4
6	100	33	4
7	33	100	33
8	4	33	100
9	0·4	4	33
10	0·04	0·4	4

Hence for a change of 0·1 pH unit the retention by $M/100$ buffer will be less than 0·5 μl. per ml. solution, if the pK' of the buffer differs from the pH of the medium by more than 2. For other values of pK' and pH the retention can be calculated from the above equation. It should be noted that the effect of temperature and salt concentration on pK' may cause a considerable alteration in retention.

Summary

The retention of acids by serum has been considered in relation to the change of pH of the serum. The "Retention Coefficient" R, representing the amount of retention per unit decrease of pH, has been used to calculate the retention of carbonic and lactic acids by serum. Methods are described for the calculation and estimation of R.

The application of this principle to the measurement of tissue metabolism in serum is described. By this means it is possible to calculate the value of the retention for any given pressure change, an advantage not possessed by other methods of determining retention.

The retention caused by the presence of substances of known dissociation constant is described.

I am grateful to Dr F. J. W. Roughton and Dr M. Dixon for useful criticism.

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XXIX. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS

LX. GRISEOFULVIN, $C_{17}H_{17}O_6Cl$, A METABOLIC PRODUCT OF *PENICILLIUM GRISEO-FULVUM* DIERCKX

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(*Received 22 December 1938*)

The following metabolic products, present in the metabolism solution of *Penicillium griseo-fulvum* Dierckx when this mould is grown under different cultural conditions and on different culture solutions, have been isolated in this laboratory: 6-hydroxy-2-methylbenzoic acid [Anslow & Raistrick, 1931], 2:5-dihydroxybenzoic acid (gentisic acid), fumaric acid and mannitol [Raistrick & Simonart, 1933] and fulvic acid, $C_{14}H_{12}O_8$, a yellow crystalline substance of at present undetermined molecular constitution [Oxford *et al.* 1935]. The purpose of the present communication is to record observations on a hitherto undescribed chlorine-containing metabolic product of *P. griseo-fulvum* which has been isolated from the mycelium of this mould and for which the name *griseofulvin* is proposed.

Griseofulvin, $C_{17}H_{17}O_6Cl$, M.P. 218–219°, is a colourless, crystalline, neutral compound giving no colour with $FeCl_3$ and containing no free hydroxyl or carboxyl groups. It is highly dextrorotatory, $[\alpha]_{5461}+417°$.

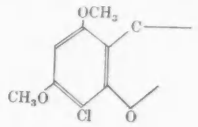
One oxygen atom is present as >CO since a crystalline mono-oxime, $C_{17}H_{18}O_6NCl$, was readily obtained; derivatives are also formed with phenylhydrazine and with 2:4-dinitrophenylhydrazine, but these could not be obtained crystalline. The function of three other oxygen atoms is obvious since griseofulvin contains three methoxyl groups. A study of the products obtained on acid and alkaline hydrolysis showed that griseofulvin must be the methyl ester of a carboxylic acid, and hence the function of a fifth oxygen atom is accounted for. The function of the sixth oxygen atom has not been definitely settled though evidence given later indicates that it is present as an oxygen bridge.

Griseofulvin, when hydrolysed with boiling *N* aqueous-alcoholic H_2SO_4, yields *griseofulvic acid*, $C_{16}H_{15}O_6Cl$, $[\alpha]_{5461}+508°$, a monobasic acid containing two methoxyl groups and giving only a feeble colour with $FeCl_3$, but these facts in themselves are not sufficient to establish the presence of the grouping $-COO.CH_3$. However, hydrolysis of griseofulvin, or further hydrolysis of griseofulvic acid, with boiling aqueous *N*/2 NaOH yields nor*griseofulvic acid*, $C_{15}H_{13}O_6Cl$, $[\alpha]_{5461}+609°$, a dibasic acid containing only one methoxyl group, together with *decarboxygriseofulvic acid*, $C_{15}H_{15}O_4Cl$, $[\alpha]_{5461}-31°$, an insoluble *neutral* compound containing two methoxyl groups, giving no colour with $FeCl_3$, and derived from griseofulvic acid by the loss of 1 mol. of CO_2. Decarboxygriseofulvic acid is stable to acid hydrolysis and hence it seems certain that griseofulvin contains only one $-COO.CH_3$ group and that the second acidic

group in *nor*griseofulvic acid is a phenolic and not a carboxyl group. This conclusion is supported by the colour reactions of *nor*griseofulvic acid, an intense brown colour with alcoholic $FeCl_3$, an intense orange brown colour with diazotized sulphanilic acid in Na_2CO_3 solution and a positive Millon reaction.

On catalytic reduction with palladium-charcoal-hydrogen, griseofulvin gives rise to two reduction products: *dihydrogriseofulvin*, $C_{17}H_{19}O_6Cl$, $[\alpha]_{5461} - 33°$, and *tetrahydrodeoxygriseofulvin*, $C_{17}H_{21}O_5Cl$. Dihydrogriseofulvin still contains the >CO group present in griseofulvin since it forms a 2:4-dinitrophenylhydrazone with Brady's reagent. Hence the two hydrogen atoms taken up in the formation of dihydrogriseofulvin must have been absorbed in the saturation of a —C=C— linkage. This conclusion is supported by the fact that whereas griseofulvin gives no coloration with Täufel & Thaler's [1932] reagent—warming with salicylaldehyde and 45% aqueous H_2SO_4—dihydrogriseofulvin gives a deep red colour with this reagent. This indicates clearly the presence in dihydrogriseofulvin of the grouping $—CH_2.CO.CH_2—$ so that griseofulvin itself must contain the grouping $—CH_2.CO.CH{=}C<$. Tetrahydrodeoxygriseofulvin contains two hydrogen atoms more and one oxygen atom less than dihydrogriseofulvin. This fact is most readily explained by assuming that the grouping $—CH_2.CO.CH_2—$ in dihydrogriseofulvin becomes $—CH_2.CH_2.CH_2—$ in tetrahydrodeoxygriseofulvin, an assumption that is supported by the fact that tetrahydrodeoxygriseofulvin does not react with Brady's reagent.

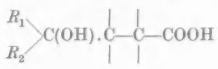
On oxidation of griseofulvin with $KMnO_4$ in acetone at room temperature two degradation products were isolated. The first of these, $C_9H_9O_5Cl$, is a phenolic monobasic acid containing two methoxyl groups. It must be a chlorohydroxydimethoxybenzoic acid since, with diazomethane, it gave methyl 3-chloro-2:4:6-trimethoxybenzoate identical with that prepared by Calam & Oxford [1939] from phloroglucinol carboxylic acid. Since the griseofulvin degradation product gives a deep purple colour with $FeCl_3$ it is clearly a derivative of salicylic acid. Hence it must be either 5-chloro-2-hydroxy-4:6-dimethoxybenzoic acid or *3-chloro-2-hydroxy-4:6-dimethoxybenzoic acid*, (VII), and since it gives a negative Millon reaction, indicating the absence of an unsubstituted carbon *ortho* to the phenolic group, it must have the latter orientation. The same degradation product is also obtained under similar conditions of oxidation from griseofulvic acid, $C_{16}H_{15}O_6Cl$, and from decarboxygriseofulvic acid, $C_{15}H_{15}O_4Cl$. These facts lead to the following conclusions: (*a*) griseofulvin itself contains a benzene ring to which are attached two of the three $—O.CH_3$ groups and also the chlorine atom; (*b*) the $—COO.CH_3$ group present in griseofulvin is almost certainly not attached to this benzene ring; (*c*) the —COOH and —OH groups in the degradation product arise respectively from the oxidation of a —C— and a —O— linkage. There is present, therefore, in griseofulvin the nucleus



So far as we are aware griseofulvin affords the first recorded instance of the occurrence of the phloroglucinol nucleus in a mould metabolic product.

That griseofulvin, in addition to the above nucleus, also contains a second ring or a long side chain attached to the benzene ring through the —C— and —O— groups, and containing one or more centres of asymmetry, is indicated by

the second degradation product referred to above. This substance, $C_{14}H_{15}O_7Cl$, $[\alpha]_{5790}-24°$, is a monobasic acid giving no colour with $FeCl_3$. It contains two methoxyl groups, a —CO— group which cannot be present as $—OC.CH_3$ since the substance does not give iodoform with alkaline iodine and a hydroxyl group which must be tertiary since no reaction is given with the Fearon-Mitchell [1932] reagent for primary and secondary alcohols. On treatment with acetic anhydride and pyridine it did not give an acetate, but the elements of water were eliminated to give a neutral substance, $C_{14}H_{13}O_6Cl$. The original substance, $C_{14}H_{15}O_7Cl$, is therefore probably a γ-hydroxy-acid of the form

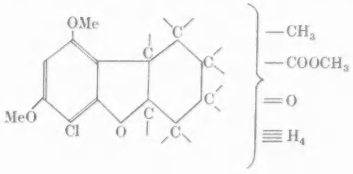


where neither R_1 nor R_2 is hydrogen.

Griseofulvin, on fusion with KOH, gives orcinol (3:5-dihydroxytoluene, IX). It is difficult, if not impossible, to explain how the substituted benzene ring which yields 3-chloro-2-hydroxy-4:6-dimethoxybenzoic acid on oxidation of griseofulvin with $KMnO_4$ at room temperature could also yield orcinol on KOH fusion. Hence the conclusion seems irresistible that 3-chloro-2-hydroxy-4:6-dimethoxybenzoic acid and orcinol arise from separate halves of the griseofulvin molecule and, as was indicated earlier, that the $—COOCH_3$ group is attached to the orcinol-yielding half.

Finally, both griseofulvic acid, $C_{16}H_{15}O_6Cl$, and *nor*griseofulvic acid, $C_{15}H_{13}O_6Cl$, with diazomethane, give not only griseofulvin, identical in its properties with the natural product, but also an *iso*griseofulvin, having the same empirical formula as griseofulvin but with a different M.P. and having $[\alpha]_{5461}+265°$ instead of $+417°$.

The experimental facts at present available do not lead with certainty to a structural formula for griseofulvin. If, however, it is accepted that 3-chloro-2-hydroxy-4:6-dimethoxybenzoic acid and orcinol arise from separate halves of the griseofulvin molecule, and if it is further assumed that orcinol arises from a preformed six-carbon ring with a methyl side-chain attached, then the skeleton



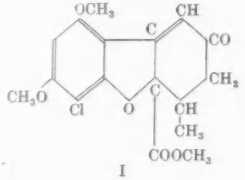
seems inevitable. The non-chlorinated ring must contain the grouping

$$>C=CH.CO.CH_2—$$

(see under dihydrogriseofulvin) and also the centre or centres of optical activity. A number of possible structures, which would satisfy the experimental facts available, could be advanced, but of these (I) appears to offer the best working hypothesis and is suggested as a tentative formula.

Accepting (I) for griseofulvin, $C_{17}H_{17}O_6Cl$, then griseofulvic acid, $C_{16}H_{15}O_6Cl$, would be (II), *nor*griseofulvic acid, $C_{15}H_{13}O_6Cl$ (III), decarboxygriseofulvic acid, $C_{15}H_{15}O_4Cl$ (IV), dihydrogriseofulvin, $C_{17}H_{19}O_6Cl$ (V), tetrahydrodeoxygriseofulvin, $C_{17}H_{21}O_5Cl$ (VI), and the $KMnO_4$ oxidation product of griseofulvin,

$C_{14}H_{15}O_7Cl$ (VIII). The known properties of these substances can be readily explained on the basis of the structures proposed for them.



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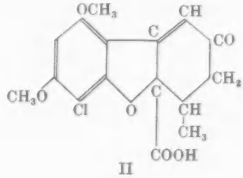

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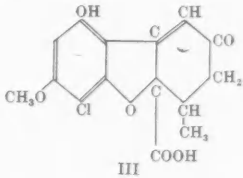

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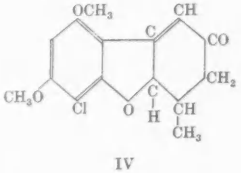

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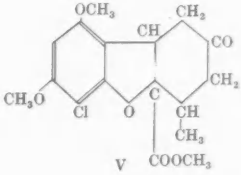

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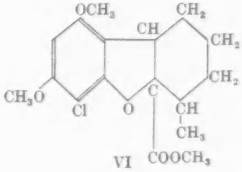

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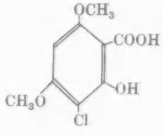

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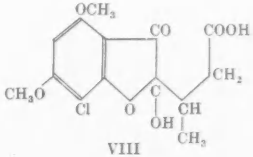

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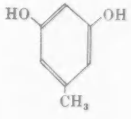

IX

Experimental

History of the culture used

The culture of *Penicillium griseo-fulvum* Dierckx used throughout this work was received in December 1929 from Prof. Ph. Biourge of the University of Louvain, and was numbered B. 34. It bears the L.S.H.T.M. Catalogue No. P. 38.

Cultural conditions

Batches of 100 flasks of the following solution (glucose, 80 g.; $NaNO_3$, 2·5 g.; KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4, 7H_2O$, 0·5 g.; $FeSO_4, 7H_2O$, 0·02 g.; distilled water, 1000 ml.), 350 ml. in each litre conical flask, were sterilized, sown with a spore suspension of *P. griseo-fulvum*, and incubated at 30° for 65–85 days when the glucose had been reduced to 0·6–0·8 %. At the end of the incubation period the mycelium was separated by filtration, washed with cold water, and dried in a vacuum oven at 50°. Weight of dry mycelium from 740 flasks = 2970 g.

Extraction of griseofulvin

The mycelium was finely ground in a coffee mill, mixed with pumice stone which had been previously washed with ether, and extracted in a Soxhlet apparatus for 3 days with light petroleum (B.P. 40–50°). The extract consisted of an oil containing small amounts of griseofulvin. The mycelium was then air-dried and re-extracted with ether for 4 days and gave 286 g. of crude solid material = 9·6 % of the dried mycelium. This crude solid material consists of a mixture of griseofulvin and a hitherto undescribed mould metabolic product containing nitrogen, which will form the subject of a future communication.

The constituents were separated as follows: 10–15 g. lots were extracted with boiling benzene (350 ml.). The benzene solution, on cooling, deposited the nitrogenous compound in a fairly pure condition. The benzene mother liquors, by evaporation in stages, yielded successive crops of griseofulvin which was purified by crystallization from ethanol. Total yield of pure griseofulvin, 48·9 g.

When *P. griseo-fulvum* was grown on the medium described above but with an equivalent amount of KBr in place of KCl, no metabolic product containing bromine could be isolated although good growth of the mould and metabolism of the glucose occurred. No griseofulvin could be detected in the mycelium of *P. griseo-fulvum* grown on the medium used by Oxford *et al.* [1935] for the isolation of fulvic acid. This medium contains glucose and ammonium tartrate as sources of C and N and KCl as source of Cl.

Analysis and general properties of griseofulvin

Griseofulvin forms massive, colourless, rhombic crystals by slow deposition from ethanol, M.P. 218–219° without decomposition. (Found: C, 58·05, 57·86; H, 4·93, 4·90; Cl, 9·98, 10·09; CH_3O, 26·29, 26·91 %; N, nil; mol. wt. (cryoscopic in dioxane), 309; (cryoscopic in camphor), 348, 361. $C_{14}H_8O_2Cl(CH_3O)_3$, i.e. $C_{17}H_{17}O_6Cl$ requires C, 57·86; H, 4·85; Cl, 10·06; $3CH_3O$, 26·40 %; mol. wt. 352·5.) The substance is quite neutral; 0·0960 g. dissolved in aqueous ethanol required only 0·05 ml. *N*/10 NaOH for neutralization to phenolphthalein. Optical rotation, $[\alpha]^{19°}_{5790}+354°$; $[\alpha]^{19°}_{5461}+417°$. ($c=1·0127$ in acetone.) It is sparingly soluble in the cold, in chloroform, ethyl acetate, benzene, toluene, ethanol, acetone and dioxane, and is quite insoluble in water. The small solubility in cold dioxane renders an exact determination of the mol. wt. impossible. It gives no colour with ferric chloride in alcoholic solution, but gives a yellow colour without fluorescence in cold conc. H_2SO_4 and a similar colour in cold conc. HNO_3. It does not decolor bromine in chloroform solution and gives no reactions with concentrated HBr in glacial acetic acid (not a γ-pyrone) or with alkaline sodium nitroprusside and pyridine (not a $\beta\gamma$-unsaturated lactone [Jacobs & Hoffman, 1926]). It cannot be acetylated. It reacts very readily with phenylhydrazine and with 2:4-dinitrophenylhydrazine but crystalline derivatives could not be isolated in a pure state. It does not react with diazomethane or semicarbazide.

Identification of the alkoxyl groups in griseofulvin. The alkyl iodide from a Zeisel decomposition of griseofulvin (0·1655 g.) was passed into dimethylaniline. The white crystalline precipitate formed (0·3590 g., theoretical for $3CH_3O$ groups in griseofulvin 0·3705 g.) behaved on heating in an exactly similar way to authentic dimethylaniline methiodide, i.e. it melted at 206° in a sealed tube and was completely volatilized at 220°. (Found: I, 48·41 %. Calc. for $C_6H_5(CH_3)_3NI$; I, 48·27 %.) All three alkoxyl groups in griseofulvin are thus methoxyl groups.

Griseofulvin mono-oxime. Griseofulvin (0·7 g.) in ethanol (20 ml.) was refluxed for 7 hr. with hydroxylamine hydrochloride (1·0 g.) and *N* NaOH (14·3 ml.). On dilution with water the crude oxime separated and was purified by repeated crystallization from benzene-light petroleum. Clusters of shining, colourless leaflets which retained solvent very tenaciously. The air-dried crystals sintered at 120° and melted with gas evolution at 120–40° (probably loss of solvent); the melt reset on further heating and remelted sharply at 226–7° without decomp. (Found, on material dried to constant weight at 110°: C, 55·33; H, 5·23; N, 3·46; Cl, 9·06 CH_3O, 25·25 %. $C_{17}H_{18}O_6NCl$ requires

C, 55·49; H, 4·92; N, 3·81; Cl, 9·64; $3(CH_3O)$, 25·31 %.) In spite of the large excess of hydroxylamine used in this preparation there was no evidence of the formation of a di-oxime.

Catalytic reduction of griseofulvin. Dihydrogriseofulvin and tetrahydrodeoxygriseofulvin

To a solution of griseofulvin (0·9 g.) in ethyl acetate (140 ml.) were added norite (2·5 g.) and a suspension of powdered $PdCl_2$ (0·7 g.) in water (50 ml.) previously heated to boiling and then cooled. The mixture was vigorously shaken with hydrogen and after the initial very rapid uptake of hydrogen required for the reduction of the $PdCl_2$ and saturation of the catalyst a slow absorption set in. The reduction was stopped after 7 hr. when 61 ml. H_2 had been absorbed (theoretical for 1 mol. H_2 = 57 ml. at N.T.P.). The mixture was filtered, the ethyl acetate layer separated, dried and concentrated to low bulk. Addition of much light petroleum now gave a colourless precipitate (0·7 g., M.P. 184–186°) from which were obtained, by fractional crystallization from light petroleum (B.P. 80–100°) and aqueous ethanol, two pure substances, dihydrogriseofulvin and tetrahydrodeoxygriseofulvin.

Dihydrogriseofulvin. Colourless flat rods, M.P. 194–196° from light petroleum (B.P. 80–100°). (Found: C, 57·60, 57·57; H, 5·31, 5·25; Cl, 10·2 %. $C_{17}H_{19}O_6Cl$ requires C, 57·53; H, 5·40; Cl, 10·0 %.) Optical rotation, $[\alpha]^{18°}_{5461} - 33°$; $[\alpha]^{18°}_{5780} - 27°$. ($c$ = 0·452 in acetone.) Dihydrogriseofulvin is more readily soluble in most organic solvents than griseofulvin. It gives only a yellow colour with $FeCl_3$ in alcoholic solution. Unlike griseofulvin, which gives no colour, it gives a deep rose red colour on warming with salicylaldehyde and 45 % aqueous H_2SO_4. Addition of Brady's reagent [1931] to an alcoholic solution gives a yellow crystalline precipitate, M.P. 264–266°, which is probably a mono-2:4-dinitrophenylhydrazone since it gives a reddish brown colour with alcohol and a trace of aqueous KOH.

Tetrahydrodeoxygriseofulvin. Long colourless slender needles, M.P. 180°, from aqueous ethanol or methanol. (Found: C, 59·99, 60·00; H, 6·28, 6·43; Cl, 10·5, 10·6; CH_3O, 26·9 %. $C_{17}H_{21}O_5Cl$ requires C, 59·88; H, 6·22; Cl, 10·4; $3CH_3O$, 27·3 %.) The substance is quite insoluble in dilute KOH. It gives an intense greenish yellow colour with cold concentrated H_2SO_4 becoming wine-red on warming. A solution in aqueous alcohol gives no precipitate with Brady's reagent even on heating. It is not hydrolysed by boiling for 4 hr. with aqueous-alcoholic N H_2SO_4 or by boiling for 7 hr. with $N/2$ NaOH, facts which are difficult to explain unless a steric hindrance effect is assumed.

Hydrolysis of griseofulvin

(A) *With dilute aqueous-alcoholic H_2SO_4. Griseofulvic acid.* A mixture of griseofulvin (1·0 g.), ethanol (200 ml.) and $2N$ H_2SO_4 (250 ml.) was refluxed for 6 hr. The ethanol was removed *in vacuo*, the colourless precipitate collected, washed with water and then with dilute Na_2CO_3 in which it was almost completely soluble. The alkaline solution was acidified and the resulting precipitate (0·8 g.) was crystallized from boiling aqueous methanol in which it is only sparingly soluble. Colourless, flat, hexagonal prisms, M.P. 256–260°. (Found: C, 56·85, 56·77; H, 4·54, 4·61; Cl, 10·38, 10·14; CH_3O, 18·2 %. Equiv. by titration, 362. $C_{16}H_{15}O_6Cl$ requires C, 56·70; H, 4·46; Cl, 10·47; $2CH_3O$, 18·3 %. Equiv., as a monobasic acid, 339.) Optical rotation, $[\alpha]^{19°}_{5461} + 508°$; $[\alpha]^{19°}_{5780} + 420°$. ($c$ = 0·2256 as the sodium salt in aqueous methanol.)

Griseofulvic acid gives a yellowish brown colour with $FeCl_3$, a yellow colour with cold conc. H_2SO_4, and only a faint yellow colour with diazotized sulphanilic acid in Na_2CO_3 solution. The Millon reaction is also negative. It is readily soluble in aqueous $NaHCO_3$ and the resulting solution slowly decolorizes very dilute $KMnO_4$ solution. It gives a dark red amorphous precipitate with Brady's reagent.

(B) *With dilute aqueous-alcoholic NaOH.* A mixture of griseofulvin (2·25 g.), ethahol (300 ml.) and *N*/4 NaOH (200 ml.) was refluxed for 3 hr. The solution was cooled, acidified, the ethanol removed *in vacuo* and the colourless precipitate collected, washed and dried (2·3 g.). It was crystallized from aqueous methanol in colourless hexagonal prisms, M.P. 255–262°, alone or in admixture with griseofulvic acid formed on acid hydrolysis of griseofulvin (see (A) above). Equiv. by titration, 374. $C_{16}H_{15}O_6Cl$, as a monobasic acid, requires 339.

(C) *With dilute aqueous NaOH.* A mixture of griseofulvin (3·82 g.), *N* NaOH (250 ml.) and water (350 ml.) was refluxed for 5 hr. At first part of the solid appeared to dissolve, but later much colourless material separated. Next day this was collected, washed and dried (fraction I, wt. 1·04 g.). The alkaline filtrate was acidified, filtered from a little dark coloured amorphous material and set aside. An almost colourless, crystalline substance (fraction II, wt. 1·6 g.) separated.

Purification and properties of fraction I. Decarboxygriseofulvic acid. Colourless needles from aqueous ethanol, M.P. 138–140°. Contains chlorine. (Found: C, 61·25, 61·15; H, 5·09, 5·10; CH_3O, 21·01, 21·37; mol. wt. (cryoscopic in dioxane), 279. Equiv. nil. $C_{15}H_{15}O_4Cl$ requires C, 61·10; H, 5·13; $2CH_3O$, 21·05 %; mol. wt. 295.) Optical rotation, $[\alpha]^{18°}_{5461} -31°$. ($c = 0·163$ in acetone.)

Decarboxygriseofulvic acid is much more soluble in the usual organic solvents than is griseofulvin. It gives no colour with $FeCl_3$ in aqueous alcoholic solution. With cold conc. H_2SO_4 it gives an immediate intense yellow colour, quickly changing to brown and finally to a deep purple (permanganate) colour. It gives a brownish red colour with saturated alcoholic picric acid but no picrate separates. It is unchanged by boiling with dilute aqueous-alcoholic H_2SO_4.

Purification and properties of fraction II. nor*Griseofulvic acid.* Long, colourless, pointed needles from aqueous methanol, M.P. 260° decomp. (Found: C, 55·67; H, 4·18; Cl, 10·46, 10·61; CH_3O, 10·6 %. Equiv. by titration, 165. $C_{15}H_{13}O_6Cl$ requires C, 55·46; H, 4·04; Cl, 10·92; $1CH_3O$, 9·6 %. Equiv. as a dibasic acid, 162.) Optical rotation, $[\alpha]^{18°}_{5461} +609°$; $[\alpha]^{18°}_{5790} +505°$. ($c = 0·2262$ as sodium salt in water.) *nor*Griseofulvic acid is readily soluble in hot methanol but is almost insoluble in boiling chloroform. It gives a positive Millon reaction, an intense brown colour with $FeCl_3$ in aqueous methanol, an intense orange brown colour with diazotized sulphanilic acid in Na_2CO_3 and a yellow colour with cold conc. H_2SO_4. It was recovered unchanged after boiling with *N* aqueous-alcoholic H_2SO_4.

(D) *Hydrolysis of griseofulvic acid with N/2 aqueous NaOH.* Griseofulvic acid ($C_{16}H_{15}O_6Cl$, ((A) above) 0·53 g.) was boiled with *N*/2 aqueous NaOH (70 ml.) for 4 hr. The products of hydrolysis were decarboxygriseofulvic acid, $C_{15}H_{15}O_4Cl$ ((C) above, fraction I, 0·13 g., M.P. 138–140° alone or in admixture) and *nor*griseofulvic acid, $C_{15}H_{13}O_6Cl$ ((C) above, fraction II, 0·15 g., M.P. 260° alone or in admixture).

Methylation of griseofulvic acid and nor*griseofulvic acid with diazomethane*

*nor*Griseofulvic acid, $C_{15}H_{13}O_6Cl$ (0·38 g.), suspended in ether was methylated by the addition of diazomethane prepared from nitrosomethylurethane (7 ml.). After several hours the solution was separated from a little insoluble precipitate,

the solvent removed and the crystalline residue (0·43 g.) fractionally crystallized from ethanol. The more soluble component proved to be griseofulvin. Prisms, M.P. 217–218°, alone or in admixture with authentic griseofulvin. The less soluble component proved to be an *iso*griseofulvin, long colourless needles from ethanol (0·15 g.), M.P. 198–200°, and giving a large depression on admixture with griseofulvin. (Found: C, 57·82, 57·88; H, 4·98, 4·99; N, nil; Cl, 10·20; CH_3O, 26·51 %. $C_{17}H_{17}O_6Cl$ requires C, 57·86; H, 4·85, Cl, 10·06; $3CH_3O$, 26·40 %.) Optical rotation, $[\alpha]^{19°}_{5461}+265°$; $[\alpha]^{19°}_{5790}+223°$. ($c=0·1424$ in acetone.) It is very similar in all its properties to griseofulvin except that it is less soluble in organic solvents.

Methylation of griseofulvic acid, $C_{16}H_{15}O_6Cl$, with diazomethane also gave a mixture of griseofulvin and *iso*griseofulvin, though in this case griseofulvin was formed in the larger amount.

Oxidation of griseofulvin by $KMnO_4$ in acetone

Griseofulvin (4 g.) dissolved in pure acetone (1 litre) was treated with finely powdered $KMnO_4$ (16 g.). The mixture was kept cool and shaken occasionally during 5 hr. The MnO_2 and insoluble potassium salts formed were separated, washed with acetone, dried and ground with dilute ammonia. The filtered ammoniacal solution was acidified to Congo red with dilute H_2SO_4. A gum was precipitated which later solidified (0·8 g.). (For treatment of filtrate from gum, see below.) The gum was repeatedly crystallized from ethyl acetate to yield 0·2 g. of colourless needles, M.P. 224° decomp., of *3-chloro-2-hydroxy-4:6-dimethoxybenzoic acid*. (Found: C, 46·52, 46·49; H, 4·01, 3·96; Cl, 15·36, 15·64; CH_3O, 26·18, 26·42 %. Equiv. 231. $C_9H_9O_5Cl$ requires C, 46·45; H, 3·90; Cl, 15·25; $2CH_3O$, 26·67 %. Equiv. as a monobasic acid, 232·5.) This acid is insoluble in water and sparingly soluble in all organic solvents. It gives a beautiful purple colour with $FeCl_3$ in alcoholic solution but gives a negative Millon reaction.

0·3 g. of the acid, on methylation with ethereal diazomethane, yielded 0·15 g. of the methyl ester of 3-chloro-2:4:6-trimethoxybenzoic acid [Calam & Oxford, 1939]. Colourless hexagonal platelets from light petroleum, M.P. 127–128°, alone or in admixture with an authentic specimen. (Found: C, 50·55, 50·40; H, 5·01, 5·04; CH_3O, 47·20, 46·79 %. Mol. wt., cryoscopic in dioxane, 248. $C_{11}H_{13}O_5Cl$ requires C, 50·67; H, 5·03; $4CH_3O$, 47·63 %; mol. wt. 260·5.) The substance is quite insoluble in KOH solution and gives no colour with $FeCl_3$.

3-Chloro-2-hydroxy-4:6-dimethoxybenzoic acid is also formed by the oxidation with $KMnO_4$ in acetone of griseofulvic acid, $C_{16}H_{15}O_6Cl$, and decarboxygriseofulvic acid, $C_{15}H_{15}O_4Cl$.

The filtrate from the gum referred to above was extracted with ether. On removal of the solvent a small amount of colourless material remained which, on crystallization from benzene-light petroleum-chloroform, yielded clusters of minute rods, M.P. 200° decomp. (Found: C, 50·70, 50·79; H, 4·48, 4·45; Cl, 10·7, 11·1; CH_3O, 18·4, 18·3 %. Equiv., 320. $C_{14}H_{15}O_7Cl$ requires C, 50·82; H, 4·57; Cl, 10·73; $2CH_3O$, 18·77 %. Equiv. as a monobasic acid, 330·5.) Optical rotation, $[\alpha]^{18°}_{5790}-24°$. ($c=0·8506$ as the sodium salt in 20 % aqueous methanol.) The acid is appreciably soluble in water, readily so in ethanol, and gives no colour with $FeCl_3$. It appears to contain a —CO— group since, although its aqueous solution does not give an immediate reaction with Brady's reagent, a good yellow precipitate forms after 2 days. It also appears to contain an OH group since, on treatment with acetic anhydride in pyridine at 37° for several days, although no acetyl derivative is formed, the elements of water are eliminated to give a *neutral substance*, $C_{14}H_{13}O_6Cl$, as colourless needles from aqueous ethanol

M.P. 220° (no decomp.). (Found: C, 53·87; H, 4·18; Cl, 11·14%. $C_{14}H_{13}O_6Cl$ requires C, 53·74; H, 4·19; Cl, 11·34%.) The acid $C_{14}H_{15}O_7Cl$ gives a negative iodoform reaction with alkaline iodine, a negative Fearon-Mitchell reaction [1932] for primary and secondary alcohols and it does not appear to contain a lactone grouping since treatment with excess NaOH for some time at 37° does not unmask additional acidity. It reduces Fehling's solution on boiling. It is also formed by the $KMnO_4$ oxidation of griseofulvic acid but not of decarboxy-griseofulvic acid.

Fusion of griseofulvin with KOH

Griseofulvin (1 g.) was fused for 1 hr. at 225–50° with solid KOH (3·5 g.) and water (1·5 ml.) in a nickel crucible. The melt was cooled and dissolved in water, and the solution saturated with CO_2 and extracted with ether. Evaporation of the dried extract gave a colourless crystalline residue (0·26 g.), which, when fractionally crystallized from light petroleum gave three fractions melting at 55, 95 and 107°. All three fractions gave the characteristic reactions of orcinol, i.e. a neutral aqueous solution giving a bluish purple colour with $FeCl_3$, a red colour with sodium hypochlorite and with ammonia and a precipitate with bromine water. A pink colour with a strong green fluorescence was observed when any of the fractions was dissolved in caustic potash and shaken with chloroform. The first fraction was hydrated orcinol (M.P. 58°) and the other two essentially the anhydrous phenol (M.P. 107°). All three fractions, on sublimation in a high vacuum at 55–60° gave a sublimate melting at 96°, not depressed on admixture with authentic anhydrous orcinol. (Found: C, 67·39, 67·38; H, 6·69, 6·40%. Mol. wt. (in camphor), 129, 130. $C_7H_8O_2$ requires C, 67·70; H, 6·49; mol. wt. 124.)

SUMMARY

Griseofulvin, $C_{17}H_{17}O_6Cl$, a hitherto undescribed mould metabolic product, has been isolated from the mycelium of *Penicillium griseo-fulvum* Dierckx grown on a modified Czapek-Dox solution. The general properties of griseofulvin are described together with a number of derivatives and degradation products. A provisional structural formula for griseofulvin is suggested which illustrates the experimental findings.

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XXX. STUDIES ON THE METABOLISM OF PYRUVIC ACID IN NORMAL AND VITAMIN B_1-DEFICIENT STATES

I. A RAPID, SPECIFIC AND SENSITIVE METHOD FOR THE ESTIMATION OF BLOOD PYRUVATE

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(*Received 19 December 1938*)

Since the appearance of the Meyerhof-Embden scheme pyruvic acid is known to be a normal intermediate metabolite of the carbohydrate breakdown of mammalian tissues, of yeast fermentation and of the respiration of higher plants. It is involved in the metabolism of embryos, bacteria and tumour tissues, and in the action of hormones and vitamins. It also forms a link between the metabolism of carbohydrate and amino-acids. For the study of pyruvate metabolism in man with relation to vitamin B_1-deficiency, however, a really suitable method has not hitherto been available.

No less than fifty descriptions of methods or modifications of previous methods for the identification and quantitative estimation of pyruvic acid have been reviewed by Wendel* [1932]. Since then numerous other methods have been described; there is the $NaHSO_3$-binding method of Clift & Cook [1932], the 2:4-dinitrophenylhydrazone method of Case* [1932], the carboxylase method for determination in blood [Westerkamp, 1933], the modification of the Neuberg-Case method [Peters & Thompson, 1934], the semicarbazide-HCl precipitation method and its modification [Hahn *et al.* 1934; 1936], the α-methylindole-HCl colour reaction for its estimation in blood [Dische & Robbins, 1934], the ceric sulphate method [Fromageot & Desnuelle, 1935], the red colour reaction of the 2:4-dinitrophenylhydrazone of pyruvic acid in alkaline solution [Jowett & Quastel, 1937], and finally the microchemical adaptation of the method of Clift & Cook for use with minute quantities of blood by de Jong & Picard* [1937].

Of all the methods mentioned above only a few are applicable to quantitative blood analysis because of the sensitivity required. Pyruvic acid is present in normal blood in extremely small amounts: below 0·6 mg./100 ml. The carboxylase method, apart from its questionable specificity, is too complicated for ordinary purposes. The ceric sulphate method is simple and rapid for estimating small quantities; but the interference of lactic acid prevents it from being adequate for analysis of biological fluids. Unfortunately neither of the two micro-methods described is really specific. The α-methylindole-HCl reaction of Dische & Robbins has been found by de Jong & Picard to be non-specific; the intensity of the colour developed varying with the concentration of the reagents and temperature. The author agrees with Peters & Thompson [1934] in finding that heating with alkali to remove substances other than pyruvic acid as described by Clift & Cook is not satisfactory when tissue or blood extracts are used. Many experiments of this type were done on beri-beri blood and the value never

* Criticisms of some of the previous methods are to be found in references marked with an asterisk.

agreed with that obtained by the elaborate hydrazone method: hence the micromethod described by de Jong & Picard may, like the estimation of bisulphite-binding substances, work well with pigeons' blood, but is not applicable to clinical studies. This leaves only the hydrazone method. It is specific, but suffers from the following disadvantages. First, for clinical purposes the procedure is complicated; secondly, the length of time required often renders it of no value to the physician, either for diagnosis or treatment, since acute beriberi patients usually die a few hours after admission; and thirdly the venous puncture necessary in order to obtain enough blood (at least 1·2 ml.) for every test may be an important obstacle to research studies on the changes in human or animal blood pyruvate. It is therefore necessary to have a method which is simple, specific, rapid and needs only small quantities of blood.

The difficulty in the quantitative estimation of pyruvic acid is the impossibility of separating it from other aldehyde or ketone derivatives present. In colorimetric reactions these compounds form colours which are liable to modify the tint. It is possible to develop a colorimetric determination of pyruvic acid in small quantities by the selection of optimal specific conditions in which the colour of the pyruvic acid hydrazone is maximal and permanent, and by the use of a light filter.

The principle is as follows: pyruvic acid is first converted into its 2:4-dinitrophenylhydrazone. This is extracted with ethyl acetate from the aqueous solution. The hydrazone of pyruvic acid is separated from the excess of hydrazine added and the other hydrazones of aldehyde or ketone derivatives by extraction with Na_2CO_3. Traces of the hydrazine or hydrazone carried over by the ethyl acetate dissolved in the aqueous Na_2CO_3 are removed with a fresh lot of ethyl acetate. The stable red colour developed by adding NaOH to the Na_2CO_3 extract of the 2:4-dinitrophenylhydrazone of pyruvic acid is determined colorimetrically, using a photoelectric colorimeter.[1] The interference of the yellow colour is eliminated by the use of a light filter.

Experimental details

Reagents required:

5 % and 10 % trichloroacetic acid,
0·1 % 2:4-dinitrophenylhydrazine in 2 *N* HCl,
Ethyl acetate, pure,
N NaOH,
10 % Na_2CO_3.

2 or 3 drops of freshly shed blood are dropped directly into an accurately weighed 10 ml. centrifuge tube containing 1·0 ml. 10 % trichloroacetic acid and previously cooled to below 0°. After mixing well, it is weighed again to $\pm 0{\cdot}1$ mg. or it may be convenient to use the 0·2 ml. special blood pipette described by Harrison [1937] for micro-sugar analysis. The precipitated protein is centrifuged down 10 min. after the blood is taken. The clear supernatant liquid is transferred quantitatively to an ordinary test tube (tube 1). The protein precipitate is mixed with 1 ml. 5 % trichloroacetic acid, and after being allowed to stand for 2 min. is centrifuged again and the supernatant liquid is transferred to tube 1. To the combined trichloroacetic extracts is added 1 ml. 2:4-dinitrophenylhydrazine solution. The mixture is allowed to stand at room temp. for not less than 10 min. It is then extracted with 2 ml. ethyl acetate and well mixed. When the two layers have separated out, the lower acid layer is quantitatively

[1] This is made by the Unicam Instrument Company, Cambridge, on the lines of the Evelyn Colorimeter [Evelyn, 1936].

removed with a fine-tipped dropper to tube 2, and is there extracted again with 1·0 ml. ethyl acetate. The upper ethyl acetate layer is carefully transferred to tube 1, and the lower layer is extracted for a third time with 1·0 ml. ethyl acetate. The clear aqueous layer is now discarded and the ethyl acetate added to tube 1. The combined ethyl acetate extract contains all the unchanged hydrazine added, and the hydrazones formed. From a burette exactly 2·0 ml. Na_2CO_3 are added to tube 2, rinsed well and the whole poured into tube 1. This is then mixed well with the aid of the same dropper and allowed to extract for at least 3 min. When the separation is completed the Na_2CO_3 layer is returned to tube 2. This extraction with Na_2CO_3 is repeated twice again with 2 ml. portions of Na_2CO_3 each time. The combined Na_2CO_3 solution in tube 2 is extracted for the last time with 1·0 ml. of ethyl acetate. The clear Na_2CO_3 extract, which may or may not be coloured yellow, depending on the quantity of the hydrazone present, is quantitatively transferred to a clean tube 3 to which 4·0 ml. *N* NaOH are added. A stable red colour develops which is determined after 10 min., using a photoelectric colorimeter with Wratten No. 62 light filter. The exact amount of pyruvic acid present in the given sample is read off from the standard curve.[1]

The standard curve is constructed as follows. A pure preparation of the 2:4-dinitrophenylhydrazone dissolved in ethyl acetate solution can be used, but a pure pyruvate solution is preferred. The pure pyruvic acid solution is prepared by three redistillations *in vacuo*, the fraction boiling at 55–60°/10 mm. Hg being collected. The clear liquid crystallizes out on standing in a freezing mixture at −4°. The crystals, carefully weighed in a weighing bottle, are dissolved in 50 vol. of ice-cold freshly distilled water. After neutralization with NaOH,

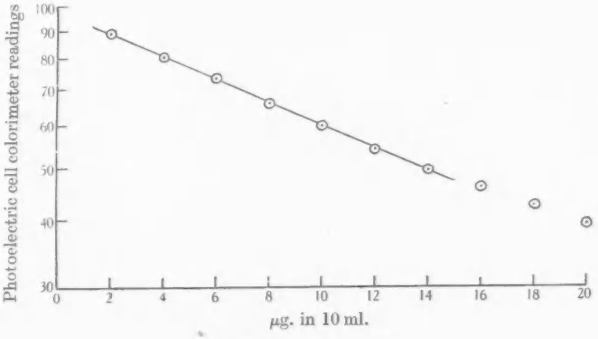

Fig. 1. Standard curve.

care being taken not to let the temperature rise, the pyruvate solution is diluted to 1%. The exact concentration of the solution is checked by estimating its bisulphite-binding power. The standard pyruvate solution thus prepared is further diluted to a concentration of 5 μg./ml. Duplicate series of tubes, containing pyruvic acid from 2 to 20 μg., are set up, and estimations carried out in the manner described above for blood analysis. The red colour developed is stable from the first min. for more than 90 min. in solutions of pure pyruvate

[1] It is always necessary to prepare a blank of trichloroacetic acid and hydrazine, extracted in the usual manner, to be used as a control solution for setting the zero of the photoelectric colorimeter. If the experiment is well carried out the blank is practically colourless every time. After each reading the galvanometer should return to the original mark.

hydrazone. The deflexions of the galvanometer readings when plotted against concentrations of pyruvic acid on arithlog paper give a straight line (Fig. 1).

The procedure just described requires a few further comments. As the method is extremely sensitive, it is essential to take every precaution to prevent loss of traces of the extract containing the pyruvic acid 2:4-dinitrophenylhydrazone by avoiding the use of a separating funnel or of numerous test tubes. We differ from previous authors, who worked on a much larger scale and in presence of a great excess of 2:4-dinitrophenylhydrazine, in finding that the Na_2CO_3 under the conditions described completely removes the pyruvic acid derivative as shown by the recovery data. Moreover, mixing gently with the aid of the dropper instead of shaking in a separating funnel gives rise to no foaming and hence no interference with the perfect separation of the two layers. Na_2CO_3 is to be preferred to Na_2HPO_4 as the latter gives a much inferior colour at the last stage. The combined techniques of extracting with ethyl acetate to remove impurities at an alkaline reaction, using a comparatively small but sufficient amount of hydrazine and developing the red colour in the aqueous Na_2CO_3 extract of the hydrazone enable us to eliminate the subsequent neutralizations and re-extractions with ethyl acetate, and the drying at the last stage, which are so laborious and liable to introduce error. 2:4-Dinitrophenylhydrazones of other keto-acids also give a red colour, but in the concentrations present in human biological fluids, whether normal or in ketosis, these substances do not interfere. Under the experimental conditions described, if they are present in larger amounts, their colour fades into yellow on standing at room temp. for 10 min. after the addition of NaOH. This reaction as used by Jowett & Quastel is not applicable to the estimation of pyruvic acid changes for studies on the physiology or pathology of animals, because the changes in blood pyruvic acid are so small [Thompson & Johnson, 1935; Platt & Lu, 1935; 1936] and the method is not specific enough.

A comparison of pyruvate values was made between the present method and the modified Neuberg-Case hydrazone method [Peters & Thompson, 1934]. The results are shown in Table I.

Table I

	mg. per 100 g.			
Specimen used	Macro-method (Neuberg-Case)	New micro-method	Difference	Micro-method % of macro-method
Blood	0·765	0·770	0·005	101
Milk	0·584	0·570	0·014	98
Cerebro-spinal fluid	0·940	0·951	0·011	101

From these figures it is obvious that the methods are in good agreement.

Sensitivity and specificity of the method

The method estimates 2 μg. in 10 ml., a dilution of 1 : 5,000,000 with an error of $\pm 1{\cdot}5\%$. It is 1000 times more sensitive than the reaction of Simon & Piaux [1924] and 50 times more sensitive than the method of Case [1932]. Since the same principle of isolating the hydrazone of keto-acids by carbonate extraction is used in the new method its specificity is at least as high as that of the Neuberg-Case method. Indeed the use of the light filter has rendered it much more specific when small amounts of other keto-acids have been added. How far this is important for practical purposes in studies of blood in diseased conditions is not yet determined.

For many years acetoacetic acid has been known to increase in the blood in ketosis. Recently Krebs [1938] has found α-ketoglutaric acid to be present in human blood and urine, while oxaloacetic acid has been found by numerous authors to play a part in tissue metabolism. It is possible that laevulic and glucuronic acids may also prove to be of physiological importance. It may therefore be necessary to determine the total amount of keto-acids present. This may be done by substituting 15% Na_2CO_3 for 10%, by using 3 *N* instead of *N* NaOH, and by taking the reading 2 min. after mixing. Under these conditions all the keto-acids give maximum colour.

On the other hand, under the conditions described in this paper, where pyruvic acid alone is to be estimated, the relative interference is as follows:

	Deflexion of the photo-cell colorimeter reading equivalent to 1 μg. pyruvic acid
Acetoacetic acid	59 μg.
Laevulic acid	15 ,,
α-Ketoglutaric acid	7·5 ,,

Oxaloacetic acid, if present, is of course always estimated as pyruvic acid, being unstable in acid solution. The total keto-acids could be estimated after a pyruvic acid determination by adding concentrated Na_2CO_3 and NaOH to make the final proportions as indicated above, and then measuring the colour again in the colorimeter. In this case, however, an appropriate calibration curve would have to be worked out.

The present method possesses yet further advantages over the standard Neuberg-Case method, for whereas the application of the latter to urine often leads to yellowish brown colours and gives purplish ones with tissue extracts, none of these inconveniences are met with in the present method.

Application of the method to the analysis of cerebro-spinal fluid, milk, urine and muscle involves no change except in the preparation of the pyruvic acid extract. Deproteinization of cerebro-spinal fluid and urine is carried out in the same manner as with blood. For tissue 8 parts of 5% CCl_3COOH are used for every g. In the case of milk, 1 ml. of 75% $(NH_4)_2SO_4$ is used in place of 1 ml. 10% and 5% trichloroacetic acid. Tables II and III illustrate the recoveries of added pyruvate in blood and milk.

Table II. *Recovery of pyruvic acid from human blood*

Pyruvic acid found in 100 ml. blood mg.	Pyruvic acid added per 100 ml. mg.	Pyruvic acid recovery	
		Found mg./100 ml.	Calc. mg./100 ml.
0·56 (normal)	2·0	2·52	2·56
0·98 (subacute beri-beri)	1·0	1·93	1·98
2·35 (acute beri-beri)	0·5	2·82	2·85

Table III. *Recovery of pyruvate from milk*

Pyruvic acid found in 100 ml. milk mg.	Pyruvic acid added per 100 ml. mg.	Pyruvic acid recovery	
		Found mg./100 ml.	Calc. mg./100 ml.
0·24	0·75	0·98	0·99
0·34	1·00	1·34	1·34
0·53	0·50	1·06	1·03

Recoveries from tissue extracts are of the same order.

SUMMARY

The colour of the 2:4-dinitrophenylhydrazone of pyruvic acid may be used for the rapid, specific and sensitive micro-estimation of this substance in biological fluids if new extraction procedures for removing interfering substances are adopted, and if a light filter is used in the photoelectric cell colorimeter to increase accuracy by eliminating accompanying traces of other tints.

ACKNOWLEDGEMENTS

During part of this period of work the author held a travelling research fellowship of the Henry Lester Institute, Shanghai. She is much indebted to Mr S. W. Cole for the loan of a photoelectric colorimeter, and to Sir F. G. Hopkins for the hospitality of his laboratory.

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XXXI. THE PROTEINS OF ELECTRICAL TISSUE

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(*Received 23 December 1938*)

As a corollary to the more general study of muscle proteins [Bailey, 1937, 2] an investigation of the proteins of electrical tissue is of especial interest, for the electrical organs of some elasmobranch fish in the genus *Raia* and *Torpedo* arise from the same embryonic rudiments as those of muscle, and earlier studies by Ewart have shown that the development of the electrical layer takes place at the expense of the fibrils. In the primitive organ of *R. radiata*, for instance, the metamorphosis to electrical tissue is only partially complete, the muscle fibrils developing into club-shaped cells bearing a richly innervated cup-like electrical layer and exhibiting the characteristic striations of skeletal muscle [Ewart, 1888]; in *R. circularis* and *R. fullonica* the electric cups are retained, while in *R. batis* they have developed into discs, and in all these three latter species the striations are no longer observable [Ewart, 1892]. It is in *R. clavata* and *Torpedo*, however, that the change is most marked, for here the electrical organs consist of cones or columns of cells separated by septa identical with those between the myotomes of muscle and each cell carries a nerve plate (i.e. the electrical layer), an alveolar layer and a mucilaginous material of high refractive index.

The metamorphosis of muscle rudiments to richly innervated discs suggests that the organ is essentially a modified muscle in which the contractile mechanism, generally considered to be the protein myosin, has disappeared, and that the reactions supplying electrical energy in the one case and contractile energy in the other are comparable. This supposition is supported by experiments of Baldwin & Needham [1937] and of Baldwin [1938] who find that the cyclical reactions of the glycolytic system, which are intimately associated with the liberation of energy in muscle working anaerobically, are catalysed by enzymes also common to *R. clavata* and *Torpedo*, the two species which they studied. To supplement this hypothesis, an investigation of the protein components and their comparison with those of skeletal muscle have been undertaken, and an attempt has been made to show whether the striations in the organ of *R. radiata* are coincidental with the presence of myosin, whether other globulins are also present and whether these latter have disappeared from the organs of *R. clavata* and *Torpedo* where metamorphosis from muscle rudiments to electrical tissue is most complete. The tissues have thus been compared with muscle with particular reference to their N partition, the physico-chemical properties and cataphoretic isoelectric point of the fractionated proteins, and in the case of *Torpedo* protein chemical analyses are presented.

Previous chemical studies are relatively sparse, and almost solely devoted to species of *Torpedo*. An investigation undertaken by J. Davy [1832] at the request of Sir Humphry Davy records for the tissue the low total solid content of 7%, whilst Matteuci [1838] gives figures of 9·2 and 10·4%; the latter found in the organ lactic acid, fatty substances, protein, traces of "gelatin", sodium

and potassium. Frerichs & Städeler [1858] discovered urea and Schultze [1859] reported creatinine, lactic acid, calcium phosphate, sodium chloride, sulphate and a mucous substance precipitated by acetic acid. The tissue insoluble in water he identified as fibrous tissue. Weyl [1881; 1883; 1887] added to this list nuclein, xanthine, hypoxanthine, cholesterol and made a detailed analysis of the ash. He prepared the mucin from the alcohol- and ether-extracted organ by treatment with dilute NaOH and subsequent precipitation with dilute acetic acid. After purification the product gave the following analysis: C, 52·5; H, 7·2; N, 13·2; S, 1·03 %. Baglioni [1906] carried out a proximate analysis of the tissue, recording a glycogen content of 0·09 % of the fresh weight. Later investigations have centred upon the phosphagen content [Kisch, 1930; Baldwin, 1933] and on the role of adenylic compounds [Baldwin & Needham, 1937; Baldwin, 1938].

Experimental

Nitrogen partition of the tissues. The methods evolved by Smith [1934; 1937] and by Reay & Kuchel [1936] for the partition of N between the proteins of skeletal muscle were adopted with minor variations. The total coagulable N (T.C.N.) was determined by mixing a weighed aliquot of minced tissue suspended in water with an equal volume of 10 % trichloroacetic acid. After centrifuging, the protein was washed 4–5 times with 5 % trichloroacetic acid until free from the non-coagulable nitrogenous substances, which, occurring mainly as urea, are present in large amounts in elasmobranch tissue. The N of the washed protein was determined by the Kjeldahl method. Another aliquot of tissue was exhaustively extracted at 0° by grinding with silver sand in presence of 7 % LiCl; the extract was separated by centrifuging, the grinding repeated at least 10 times, and the combined extracts were adjusted with salt solution to a volume containing 0·02–0·04 g. N/100 ml., and the protein in aliquots of this solution was determined by precipitating with trichloroacetic acid as before. Other portions of the tissue in certain instances were exhaustively extracted with *N*/20 NaOH and *N*/50 HCl.

In the case of skeletal muscle, the proteins dissolving in the LiCl solution represent the major portion of the intracellular globulins and albumins, *viz.* myosin, globulin X and myogen, but the residual intracellular fraction which, according to Smith [1937], consists mainly of denatured globulin X, dissolves, together with the salt-soluble intracellular fraction, in dilute acid or alkali; the protein undissolved by these latter is the muscle stroma. Myosin is estimated by dilution of the LiCl extract with 20 vol. of ice-cold water and the gelatinous precipitate which settles out overnight at 0·07–0·08 *M* LiCl is centrifuged off, washed with water and digested in the Kjeldahl flask. The non-coagulable N has been estimated in the LiCl and other extracts by subtracting the protein-N from the total N of the extract.

In Table I a detailed comparison between the N partition of the skeletal muscle and that of electrical tissue is made for three separate specimens of *Torpedo* treated immediately after death. For the muscle, 81–83 % of the T.C.N. is soluble in LiCl and of this 65–71 % occurs as myosin; of the salt-insoluble residue (17–19 % of the T.C.N.) a further 5–10 % is included in the acid or alkali extracts. The stroma fraction of 7–12 % is considerably higher than the 3–5 % reported by Reay & Kuchel [1936] for haddock muscle but there is as yet no systematic evidence that this fraction is constant in different muscles. The other figures presented here are in good agreement with the analyses of these authors.

Table I. *Distribution of N in g./100 g. wet tissue*

	Muscle			Electrical tissue						
	T. marmorata (1)	*T. marmorata* (2)	*T. ocellata* (3)	*T. marmorata* (1)	*T. marmorata* (2)	*T. ocellata* (3)	*R. clavata**		*R. radiata**	
Dry weight	23·2	23·8	26·2	10·8	10·6	11·8	10·4	10·0	12·0	12·8
Non-coagulable N	1·66	1·74	1·72	1·75	1·66	1·68	1·06	1·07	0·47	0·51
Total coagulable N	2·345	2·534	2·828	0·39	0·36	0·44	0·818	0·80	1·51	1·52
Coagulable N:										
In LiCl extract	1·89	2·11	2·34	0·25	0·22	0·314	0·333	0·30	0·65	0·67
In *N*/20 NaOH extract	2·09	—	—	0·27	0·227	0·34	—	—	—	—
In *N*/50 HCl extract	—	2·35	2·48	0·175	0·146	0·20	—	—	—	—
Myosin	1·67	1·65	1·90	Nil	Nil	Nil	Nil	Nil	0·15	0·09

* T.C.N. of *R. clavata* muscle 2·63; *R. radiata* muscle 2·41. Dry weight of *R. clavata* muscle 23·4%; *R. radiata* muscle 19·5%.

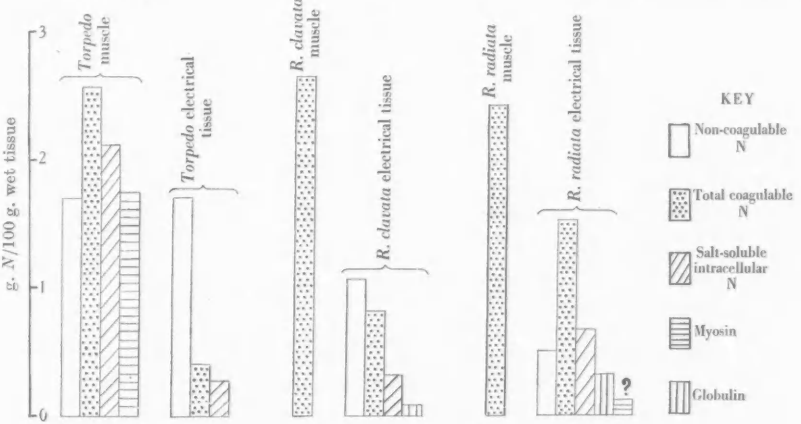

Fig. 1.

In comparison, it is seen that the dry weight of *Torpedo* electrical tissue is $<\frac{1}{2}$ and the T.C.N. only $\frac{1}{6}$–$\frac{1}{7}$ that of muscle and of this total protein, 61–77% is soluble in salt, 63–77% in *N*/20 NaOH and 40–45% in *N*/50 HCl. The comparatively large deviation in the amount of extractable coagulable N between individual organs of different specimens has been confirmed many times and may be explained by an observation of Ewart that the discs of connective tissue increase in diameter with age and decrease in thickness, whilst the amount of "gelatinous insulating material"—shown later to be a mucin—is considerably augmented; the proportion of intracellular N to stroma-N might therefore depend upon the age of the specimen. The figures reveal, however, that whereas in individual specimens, salt or alkali are equally effective in extracting the intracellular protein, the coagulable N extracted by acid is far lower, and in this respect the system differs fundamentally from muscle. For detection of myosin, it is not possible to utilize the sol obtained by exhaustive extraction of the tissue since the protein concentration is too low. More concentrated extracts obtained by extracting 1 part of tissue with 1 part of 14% LiCl have never yielded a precipitate. After 20-fold dilution with water, the faint opalescence which appears is due most probably to a small amount of apparent

globulin which precipitates when the LiCl sol is dialysed to a molarity of 0·03. This precipitate, which is small in comparison with the protein remaining in solution, is not a characteristic tissue globulin since it does not coagulate on heating.

The dry weight of the electrical tissue of *R. clavata* is about $\frac{1}{2}$, and the T.C.N. approximately $\frac{1}{3}$ that of the corresponding muscle; only 37–41 % of the total protein is soluble in LiCl, and salt extracts containing 0·5 g. N/100 ml. showed only a faint turbidity when diluted with 20 vol. of water. Although myosin thus appears to be absent, a heavy globulin precipitate was obtained on dialysis to a salt molarity of 0·002. The protein remaining in solution did not precipitate when the salt concentration was further reduced. The amounts of globulin obtained from various LiCl extracts bear a constant ratio to the amount of coagulable N in the extract (Table II).

Table II

	(*a*)	(*b*)	(*c*)
Coagulable N extracted by LiCl as % of T.C.N. (*A*)	31·4	35·4	35·4
Coagulable N in globulin fraction as % of (*A*)	26·4	27·8	26·3

Mean coagulable N of globulin fraction = 26·8 % of the salt-soluble protein or 9 % of the T.C.N.

The comparatively large amount of salt-insoluble protein in this tissue is to be expected from the known existence of relatively dense septa of connective tissue separating the electric discs.

Experiments with the electrical tissue of *R. radiata* were greatly handicapped by the small size of the organ, 100 dissections yielding about 6·2 g. of wet tissue. The dry weight of the organ is somewhat higher than that of previous tissues and the T.C.N. now reaches $\frac{3}{5}$ the value of *R. radiata* muscle. About 44 % of the T.C.N. is extractable by LiCl, and on dilution to a salt molarity of 0·07 a myosin-like precipitate amounting to 6–10 % of the T.C.N. separates. This precipitate, after centrifuging, was largely denatured and did not give the strong elastic film characteristic of myosin proper; moreover, the myosin prepared from *R. radiata* muscle did not denature under comparable conditions. Dialysis of the LiCl extract to lower salt concentrations (0·004 *M*) yielded an abundant globulin precipitate which was heat-coagulable. The total globulin precipitated on complete dialysis, determined in two separate extracts, amounted to 21 % of the T.C.N. and the amount of globulin salted out by saturation with NaCl amounted to a comparable figure of 19 %.

Thus of all the electrical tissues examined, that of *R. radiata* most closely resembles the protein system obtaining in muscle not only in the amount of T.C.N. but also in the comparatively large proportion of globulin, some of which precipitates at a salt molarity usually adopted for separation of myosin, but which has not been identified as myosin proper. When the results are represented diagrammatically (Fig. 1) the comparison between muscle and electrical tissue is seen to advantage. It may also be noted from this diagram that the non-coagulable nitrogenous constituents are highest where the T.C.N. is least, and in *Torpedo* amount to 1·7 % of the wet weight of the electrical organ.

General properties of electrical tissue proteins. The quantitative results indicate that all types of electric tissue contain a water-soluble protein; in *Torpedo* the whole of the salt-soluble protein is also water-soluble and in *R. clavata* and *R. radiata* a water-soluble fraction with properties similar to those of *Torpedo* protein is obtained after removal of globulins by complete dialysis. The dialysis liquors have a *p*H of approximately 7, and at this reaction the water-soluble protein is not coagulated by heat, is not immediately precipitated by $\frac{1}{2}$ saturated

$(NH_4)_2SO_4$, saturated NaCl or by addition of 1–2 vol. of alcohol. The globulins on the other hand precipitate under these conditions and are heat-coagulable. The water-soluble fraction is precipitated at *p*H 4·5–5 and in this range it can be denatured by heating; at lower *p*H values it passes into solution and on neutralizing it again precipitates at the point of minimum solubility and redissolves at *p*H 7·5. In marked contrast to these properties the water-soluble fraction (myogen) of mammalian muscle, investigated by Smith [1937], and of *R. radiata* muscle, investigated by the author, is heat-coagulable, and although it denatures on acidification in absence of salt it does not precipitate until the solution is neutralized. The properties of the water-soluble proteins of electrical tissue are not unlike those of some mucins and nucleoproteins and the preparation of the solid protein from *Torpedo* was undertaken to characterize it more completely.

Preparation of Torpedo *protein.* The minced tissue was extracted with an equal volume of water or of 14% LiCl, stirred mechanically for 1 hr. and centrifuged. The extraction was once more repeated and the combined extracts were dialysed at 1° against distilled water. After centrifuging away a small amount of precipitate, the liquid was filtered through paper pulp, acidified to *p*H 4·5 and the supernatant was decanted from the protein precipitate which was now dissolved at *p*H 7·5, centrifuged, and precipitated as before. It was dried thoroughly in alcohol and ether, and a chance evaporation of these liquors revealed the presence of lipoid which is discussed in a later section.

Two main samples of protein were analysed (Table III), *A* being prepared from a LiCl extract and *B* from a water extract.

Table III

Figures given are in percentages of total (ash free) dry wt.

	A	*B*
Ash	0·6	1·2
N	14·4	14·5
Amide-N	1·21	1·23
P	0·27	0·18
Carbohydrate	~6·5	~6
Glucosamine	3·5	3·8
Total S	1·38	1·33
SO_4-sulphur split off on hydrolysis by HCl	0·47	0·49
Cystine-S (differential oxidation)	0·40	0·39
Methionine-S (by difference)	0·51	0·45
Tyrosine	3·9	—
Tryptophan	1·32	—

The methods adopted in this analysis are described elsewhere: amide-N, total S, tyrosine and tryptophan [Bailey, 1937, 1]; P [Fiske & Subbarow, 1925]; cystine-S by differential oxidation [Lugg, 1938]; carbohydrate, the method of Tillmans & Philippi [1929] adapted by Pirie [1936]; glucosamine, the Zuckerkandl & Messiner-Klebermass method [1931] modified by Hewitt [1938].

The presence of carbohydrate, glucosamine, and of ethereal sulphate split off after hydrolysis with 5*N* HCl at 100° for 12 hr., confirms the inference, derived from physico-chemical tests, that the protein belongs to the class of mucins. The P appears also to be an integral part of the protein; it is not split off by dilute *N*/70 HCl in the cold, and only 26% is freed by digestion with *N* NaOH at 100° for 5 min. A P-rich residue was obtained in the following way: 1·58 g. of protein (P, 0·3%) were digested with 10 ml. of 0·5% pepsin solution in 30 ml. *N*/70 HCl at 27° for 2 days. The undigested sediment after washing

and drying amounted to 23% of the original protein and gave on analysis: ash, 1·0; N, 13·6; P, 0·8; S, 1·6%. Whether this residue is a nuclein cannot categorically be affirmed since attempts to isolate nucleic acid from the amount of material available have failed.

Similar proteins have been described in the literature; eel-slime, investigated by Müller & Reinbach [1914], has similar physico-chemical properties and gives a comparable analysis (N, 14·0–14·4; S, 1·16–1·29; P, 0·33–0·49%) and in its natural state is associated with lipoid. The "nucleoalbumin" isolated by Hammarsten [1885] from snail liver possesses similar properties, giving on analysis, N, 14·3; S, 1·06; P, 0·42%; when digested with pepsin a nuclein containing 2·1% P was obtained. In *Torpedo* extracts it was at first suspected that the P might arise from the abundant nuclei of the electrical layer, the isolated protein thus consisting of a mixture of mucin and nucleoprotein. Since, however, the P occurs in Müller & Reinbach's protein, which is an external skin secretion, it is unlikely that such admixture is possible, and although the P reacts as nucleic acid-P, its exact assignation to a definite prosthetic group must await further research.

Isoelectric points of electrical tissue proteins. These were determined by the cataphoretic method of Dummett & Bowden [1933] using 0·01 *M* phthalate buffers. Dried and undried preparations of proteins were ground with a little water and lightly centrifuged; aliquots of the particle suspension in the supernatant were added to a buffer series and the approximate velocity of migration was plotted against the *p*H value of the solution, measured after completion of the experiment by the hydrogen electrode. The values are given in Table IV.

Table IV

	Isoelectric point
1. *Torpedo* mucin, dried preparation, fat-free	3·15
2. *Torpedo* mucin + fat, undried	3·1
3. *R. clavata* mucin, dried preparation, fat-free	3·5
4. *R. clavata* mucin + fat, undried	3·6
5. *R. clavata* globulin, dried, fat-free	4·1
6. *R. clavata* globulin + fat, undried	4·1
7. *R. radiata* mucin + fat, undried	3·55
8. *R. radiata* mucin + fat, undried	3·6
9. *R. radiata* globulin, dried, fat-free	4·1
10. *R. radiata* myosin (from muscle)	4·6

Note on the preparations listed in Table IV. The globulin fractions 5 and 6 were obtained by dialysis of the tissue extracts and were washed thoroughly with water; their purification was not undertaken as they were partly denatured; globulin 9 was obtained by saturation of the LiCl extracts with NaCl. The mucins 3 and 4 were prepared after removal of globulins by dialysis, the residual liquors being filtered through paper pulp and acidified; the precipitated protein was then purified by redissolving at *p*H 7·5 and reprecipitating twice. The mucin of *R. radiata* was more difficult to prepare, owing to contamination of the dialysate with colloidal globulin; preparation 7 was obtained by saturating the dialysate of the LiCl extract with NaCl when a small amount of globulin separated and, after dialysis of the residual liquor, the protein was precipitated by acidification; preparation 8 was obtained by saturating the LiCl extract with NaCl and, after removal of the globulin by centrifuging and of NaCl by dialysis, the residual liquor on boiling gave a slight coagulum, which was filtered off and the mucin precipitated by acidification. The myosin (10) was prepared by the Edsall [1930] method and twice precipitated from salt solution.

Within 0·1–0·2 *p*H unit from the designated isoelectric point, differences in the direction of migration could sometimes be observed only in the case of the globulin fractions, indicating, as indeed is most likely, some degree of non-homogeneity. On the other hand, the isoelectric point of the mucin fractions was very sharp, and the low values obtained confirms their assignation to this group of proteins. The globulin fractions in bulk have an isoelectric point lower than that of *R. radiata* myosin, which in turn is lower than the reported values for that of mammalian muscle. The isoelectric point of the protein-lipoid complex does not differ greatly from that of the alcohol-extracted protein.

The lipoid material associated with electrical tissue proteins. All globulin and mucin fractions prepared from salt or water extracts of electrical tissue contain considerable amounts of lipoid which can be separated by extraction with hot alcohol and ether. After evaporation of the extracts, the residue was freed from traces of protein by re-extracting with chloroform. The amount of lipoid associated with various preparations of protein was investigated only in the case of *Torpedo* mucin, and this was not constant; e.g. the lipoid contents as % of six lipoid-protein preparations were 15·2, 14·0, 22·4, 23·0, 23·0, 14·9 % respectively.

The combined lipoid fractions from several preparations of *Torpedo* mucin were combined, re-extracted with chloroform, evaporated and dried at 100° to constant weight. The material contained 1·92 % P and 1·4 % N. The free cholesterol was estimated in the usual way by precipitation with digitonin, and the cholesterol as ester, after saponification with *N* sodium ethoxide and removal of unsaponifiable material. The free fatty acids were next extracted and weighed and the contents of phosphatides and glycerides present in the original lipoid fractions were then calculated on the assumption that the P was due exclusively to lecithin, and the fatty acids of both to oleic acid. The following figures were thus obtained:

Phosphatide	50·6 %
Free cholesterol	19·4 %
Unsaponifiable matter other than cholesterol	10·0 %
Glycerides	7·1 %
Total	87·1 %

The amount of cholesterol ester was insignificant.

It is of immediate chemical interest to consider the function of lipoid in its association with mucin, since this combination, found also in eel-slime, is probably of general occurrence in the fish mucins. It appears to act as a dispersing agent for the protein since its removal by alcohol extraction leads to complete insolubility of the protein above and below its isoelectric point, and although the denaturing action might be ascribed to the use of organic solvents, it is significant that the original protein complex is not affected by the stronger denaturing actions of heat and acid. In its native state the complex has the solubility characteristics of the mucoids, but this term has been avoided because it has not been possible to assess how much the lipoid fraction contributes to the solubility of the protein *per se*. Although the mucin-lipoid complex is stable in neutral solution, those of the globulins lose their solubility in salt after dialysis, and the globulin-LiCl extracts denature slowly on standing. These phenomena are probably due to the denaturing action of phosphatide acting in the manner described by Hardy & Gardiner [1910] for the plasma proteins and resulting, in the view of Chick [1914] in the production of euglobulin from pseudoglobulin.

SUMMARY

1. Since the electrical and muscular tissues of elasmobranchs arise from the same embryonic rudiments, a comparative study of the protein systems of the electrical tissue of *Torpedo*, *Raia clavata* and *R. radiata* has been made to ascertain whether such systems are comparable with those of muscle.

2. The N-partition of *Torpedo* muscle and of the three types of electrical tissue has been determined. Quantitatively, electrical tissue is characterized by a low total solid and low protein content; the amount of total coagulable N is highest in *R. radiata*, decreasing progressively in *R. clavata* and *Torpedo*, and the proportion of this N existing as globulin decreases in the same way. In *Torpedo* the extractable protein is almost entirely a mucin and this is common to the other electrical tissues examined.

3. The cataphoretic isoelectric points of the globulin fractions and the mucins have been determined.

4. All protein fractions are associated with comparatively large amounts of lipoid, which was isolated from *Torpedo* mucin and analysed. It consists mainly of phosphatide, free cholesterol and glyceride.

5. A partial analysis of *Torpedo* mucin is presented. It is shown to contain ethereal sulphate, carbohydrate and glucosamine, and non-labile P, which is obtained in a P-rich residue after digestion with pepsin. Attempts to isolate nucleic acid have failed.

6. The results support the view that electrical tissue, whilst retaining the enzymes associated with the glycolytic system of skeletal muscle, differs mainly in the absence of myosin, which is no longer necessary for contraction. The protein system is most dissimilar from muscle in those tissues which, from the histological standpoint, are farthest removed from muscle (e.g. *Torpedo*) and most similar in incompletely metamorphosed tissue (e.g. *R. radiata*).

I am indebted to Dr W. T. Astbury for suggesting this research, and to Prof. A. C. Chibnall, Dr E. C. Bate Smith and Dr G. A. Reay for their interest and help; to Dr G. R. Tristram and Mr M. W. Rees for assistance with some experiments; to the Marine Biological Station, Plymouth, and the Marine Laboratory, Torry, for supplies of *Raia*; to the British Association, the University of Cambridge, and to Dr R. Dohrn and staff, for the facilities provided at the Stazione Zoologica, Naples; finally to the Rockefeller Foundation for a grant.

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XXXII. A COLORIMETRIC REACTION FOR THE QUANTITATIVE ESTIMATION OF NICOTINIC ACID

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(*Received 13 December 1938*)

Now that it is known that nicotinamide is a component of cozymase, and that nicotinic acid possesses a curative effect on pellagra, a method by which this substance could be determined qualitatively and quantitatively, and which would be suitable for serial work, would be valuable.

Methods of analysis have been published previously by Swaminathan [1938], Karrer & Keller [1938], Vilter *et al.* [1938] and quite recently by Shaw & Macdonald [1938]. None of these methods is based on a specific reaction with nicotinic acid; they are founded on a colorimetric reaction of the pyridine ring.

Swaminathan's and Shaw & Macdonald's methods are based on the observation made by König [1904], that pyridine with cyanogen bromide and a primary or secondary aromatic amine develops a colour, which, according to the amine employed, varies from yellow to violet. The chemical reactions leading to colour formation have not yet been fully elucidated. Kulikow & Kresdowosdwigenskaja [1930] worked out a method based on this for determining small amounts of pyridine using aniline. Karrer & Keller [1938] and Vilter *et al.* [1938] based their work on the observation of Vongerichten [1899] who found that a colour reaction ensues when pyridine reacts with 2:4-dinitrochlorobenzene and alkali hydroxide.

Up to the present none of these methods has proved quite satisfactory. Swaminathan [1938], who as mentioned used aniline, extracted the resulting colour by shaking with *iso*amyl alcohol. In our hands this method has yielded unsatisfactory results; the colour developed is inconstant and it is difficult to obtain pyridine-free *iso*amyl alcohol.

We have not had the opportunity to test Shaw & Macdonald's [1938] method of analysis, as this appeared after we had completed the experimental part of this work. The authors, however, state that the intensity of the colour is not constant at any time, which renders their method unsuitable at any rate for serial analyses. The methods described by Karrer & Keller [1938], and by Vilter *et al.* [1938], appear to be laborious and tedious, and the authors mention that the colour obtained is very unstable.

In the present work, an account is given of a colour reaction based on König's principle. It is rapid to carry out, is performed in aqueous solution and gives a constant colour.

As colour-producing amine we have tried Na sulphanilate, Rodinal (*p*-aminophenol) and sulphanilamide (*p*-aminobenzenesulphonamide), which however gave inconstant results, whereas we found that metol (*p*-methylaminophenol sulphate) with nicotinic acid and CNBr in aqueous solution yields a clear yellow colour which, under the conditions used by us, is perfectly constant, very stable and of an intensity directly proportional to the amount of nicotinic acid.

I. Determination of nicotinic acid in colourless aqueous solution

Technique

A measured amount (up to 9 ml.) of the aqueous solution of nicotinic acid to be analysed (containing from 0·005 to 0·25 mg. nicotinic acid) is run into a graduated 20 ml. flask. After 5 min. heating on a water bath at 75–80°, 1 ml. 4 % aqueous CNBr is added. The mixture is placed on the water bath for 5 min. and then cooled under the tap to room temperature. 10 ml. saturated aqueous metol (about 5 %), and distilled water to make up the volume to 20 ml., are added. After standing 1 hr. at room temperature excluded from light, the strength of the colour developed is read off with a Pulfrich photometer (filter S. 43), with a blank solution containing the same amounts of CNBr and metol plus distilled water to make the volume 20 ml. in the other cell.

A. *The reagents*

1. *Cyanogen bromide.* We have used CNBr supplied by Fraenkel & Landau, Berlin-Oberschöneweise. A 4 % aqueous solution is employed, and it must be prepared on the day of use.

A solution of CNBr prepared as described by Kulikow & Kresdowosdwigenskaja [1930] may also be used. To a saturated aqueous solution of Br, KCN is added just to decoloration. This solution contains about 5 % CNBr.

2. *Metol* (*p-methylaminophenol sulphate*); $(CH_3NHC_6H_4OH)_2, H_2SO_4$. The metol employed is the usual product supplied by Agfa. An aqueous solution, saturated at room temperature, i.e. *c.* 5 %, is used. Thorough shaking is necessary as the metol dissolves rather slowly and the solution must not be heated. It is protected from light and must be used within 2–3 hr. of preparation. Either on heating or on further standing the solution turns brownish-violet.

B. *The analysis*

1. *The reaction between nicotinic acid and CNBr*

(*a*) *Influence of temperature.* At room temperature the reaction between nicotinic acid and CNBr proceeds very slowly. At 75–80° the reaction takes place so quickly as to be completed in about 3 min. If the mixture is heated to this temperature for more than 20 min. the compound first formed slowly disintegrates.

(*b*) *The amount of CNBr.* At least 30 mg. CNBr must be used for amounts of nicotinic acid up to 0·25 mg. Larger quantities (up to 80 mg.) do not change the extinction constant; but smaller quantities give too low an extinction value.

2. *The reaction with metol of the compound formed by nicotinic acid and CNBr*

(*a*) *Amount of metol.* The extinction constant increases with the concentration of metol in the solution until a certain point is reached. For metol quantities of 500–650 mg. in a volume of 20 ml. (i.e. from 10 to 13 ml. 5 % solution) the extinction is constant (see Fig. 1). The quantity of metol determined in this way appears rather large in relation to the amount of nicotinic acid. On recrystallization of the metol exactly the same results were obtained; thus the colour reaction can hardly be ascribed to impurities in the reagent. The final intensity of the colour depends on the metol concentration and not on the absolute quantity of metol: with a certain amount of nicotinic acid plus 250 mg.

metol diluted to a total volume of 10 ml. the colour intensity will be exactly twice that resulting from the same amount of nicotinic acid plus 500 mg. metol in a volume of 20 ml.

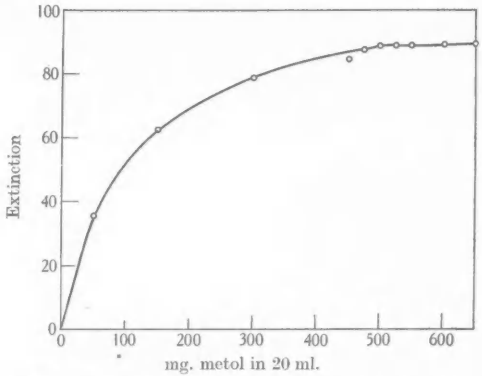

Fig. 1. Extinction obtained from 0·1 mg. nicotinic acid with 1 ml. 4 % CNBr and varying amounts of metol in a volume of 20 ml.

(*b*) *Time of reaction with metol.* On addition of metol a faint yellow coloration appears immediately and quickly intensifies. The intensity of the colour, however, first reaches its maximum after ½–1 hr.

(*c*) *Constancy of the colour developed.* At room temperature with protection from light the colour of the solution remains unchanged after 72 hr.

(*d*) *The effect of light.* If, after metol has been added, the mixture is kept in full daylight, the colour developed is fainter than if the solution has been kept in darkness; continuous exposure will result in gradual fading. However, the sensitiveness to light is not so great that the colour fades appreciably in diffuse daylight in 5–10 min.

(*e*) *The temperature.* No change occurs in the colour produced if the solution, after adding metol, is kept for 1 hr. at temperatures ranging from 5 to 30°. At 75–80° the colour developed is considerably fainter, beginning as a clear yellow it subsequently, after 20–30 min., turns more brownish-red.

3. *Photometric determination*

(*a*) *Colour.* The colour developed is a clear yellow. The extinction corresponding to the various filters of the Pulfrich photometer are approximately (0·1 mg. nicotinic acid in 1 ml. layer): S. 43, 0·89; S. 45, 0·48; S. 47, 0·26; S. 50, 0·05; S. 53–S. 75, 0·00.

(*b*) *Measurement.* With cells of 2·5–50 mm., it is possible to determine nicotinic acid in quantities ranging from 0·005 to 0·25 mg. Larger quantities yield an extinction which is too high to be measurable in a layer of 2·5 mm. In case the colour is too intense, diluting the solution with water before measurement is without avail, as this lowers the concentration of metol, thus changing the extinction constant. A fresh analysis must be performed on a more diluted solution of the sample to be examined.

(*c*) *Blank.* The blank is made with distilled water in place of the nicotinic acid solution. Blanks give a very pale pink colour. It has been observed that

CNBr preserves metol from changing to the coloured substances mentioned above; even after standing for several days in full daylight the colour of the blank does not change perceptibly.

Using the filter (S. 43) employed in the analysis in a layer of 1 cm. the blank analysis with distilled water gives an extinction which, reckoned from the moment of adding metol to 24 hr. later, gradually increases from 0·02 to 0·04.

4. *Determination of the extinction curve*

With known amounts of nicotinic acid the extinctions given in Fig. 2 have been found. Within the limits of the quantities measured the colour developed is directly proportional to the amount of nicotinic acid.

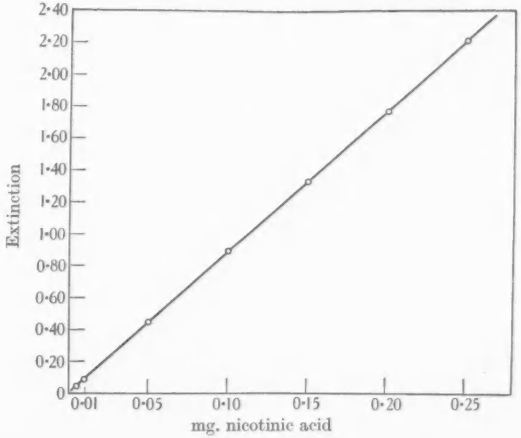

Fig. 2. Calibration curve of nicotinic acid.

Under the conditions mentioned, we have not found any variation in the extinction constant; nevertheless, as a control in each series of analyses, we have determined the value of a standard solution with known content of nicotinic acid (0·1 mg.). This has, in addition, served as a control on the reagents.

For working out the standard analysis 1 ml. of a 1 : 10,000 solution of nicotinic acid may suitably be used. In this feeble concentration the nicotinic acid is stable for a few days only. In a concentration of 1 : 1000, and in a brown glass receptacle, nicotinic acid is stable for several months at least.

5. *The sensitiveness and accuracy of the method*

As mentioned, quantities of nicotinic acid as small as 0·005 mg. may be determined with great accuracy.

It is difficult to give the percentage error. After numerous tests we believe that when pure aqueous solutions of nicotinic acid are used the error of the method is derived chiefly from the error in the photometric readings.

The method of analysis described has been worked out with pure aqueous solutions of nicotinic acid. An equiv. wt. of 122·4 (calculated 123·05) was found for the nicotinic acid used (J. D. Riedel-E. de Haën, A. G.); M.P. 232°, as stated in the literature.

The colour reaction does not tolerate free strong acid or base. In the presence of acetate ions a red-brown colour develops. Sulphate (in higher concentration than it occurs in metol) and nitrate ions cause no change in the tint, but weaken the intensity of the colour.

NaCl, NH_4Cl and KH_2PO_4 cause no change in either the tint or intensity of the colour.

If the reaction occurs in presence of acetone or alcohol, the reading is lower and inconstant.

Nicotinamide

If nicotinamide is examined by the process described above, the same results are not obtained as for equivalent amounts of nicotinic acid. The same clear yellow colour is produced, but it is usually considerably stronger, and the results are not reproducible. Therefore if nicotinic acid occurs partly or wholly as amide, it must be hydrolysed before the analysis.

II. Determination of nicotinic acid in organic material

In biological material, nicotinic acid occurs—at any rate to a large extent—in the form of the amide as a component of cozymase or Warburg's coferment; prior to determination it must, therefore, be liberated and the nicotinamide hydrolysed. We have found that this can be accomplished by heating with NaOH: as it appeared that the same result was obtained by heating on a boiling water bath for $\frac{1}{2}$ hr. as by heating in the autoclave for periods up to 2 hr. at 120°, followed by heating with 2*N* HCl (Table I), the first procedure was adopted.

Table I

Treatment	Dried yeast. Nicotinic acid found mg. %	Dried yeast + 50 mg. nicotinic acid (as amide) per 100 g. Total nicotinic acid found mg. %	Added nicotinamide found (as nicotinic acid) %
On boiling water bath for:			
30 min.	53·2	—	—
60 min.	54·2	105·7	103
90 min.	53·4	—	—
120 min.	53·2	—	—
Heating in an autoclave at 120° for:			
30 min.	53·2	102·5	99
60 min.	53·0	103·5	101
120 min.	53·2	97·6	89

Nicotinic acid added to the samples was in every case recovered quantitatively by these methods. This process results in a highly coloured homogeneous mass, which must be rendered quite colourless before it is suitable for colorimetric determination.

The removal of the colour by adsorption with charcoal or Fuller's earth at different reactions was unsuccessful, as the nicotinic acid is adsorbed with the coloured substances, and cannot be entirely eluted separately. As even filter paper adsorbs nicotinic acid, filtering must not take place at any stage of the analysis. Nor was precipitation of the coloured substances with heavy metal salts and subsequent removal of the metal ions with H_2S satisfactory, while $HgCl_2$ and Ag_2SO_4 did not precipitate the coloured substances. Pb acetate and $Hg(NO_3)_2$ cannot be used as the anions of these salts interfere with the colour

reaction. The best result, although still not satisfactory, was obtained by precipitation with $CuCl_2$. Evaporation to dryness and subsequent extraction with benzene and acetone were discarded, as neither the preformed nor the added nicotinic acid could be extracted quantitatively after drying.

However, it was found that the coloured substances could be precipitated from an aqueous phase by a large excess of acetone, in which nicotinic acid is relatively easily soluble. We have employed this procedure to remove the colour. Based on these experiments, we have worked out a method of analysis. As an example, the technique used in examining dry yeast will be described in the following.

Technique

10 ml. 2 *N* NaOH are run into a 20 ml. graduated flask without moistening its neck, and 5 g. of dry yeast are added (the order must not be reversed as the yeast will then collect in lumps which will merely be attacked superficially). The neck of the flask is closed with a wad of non-absorbent cotton wool. After shaking, the flask is placed on a boiling water bath for 30 min.; the yeast will then be fully dissolved. After cooling, concentrated 36 % HCl (1·8 ml.) is added drop by drop, and the mixture thoroughly shaken. The reaction will now have become slightly acid (pH=4–6). The solution is cooled to about 20°, and distilled water is added to make up the volume to 20 ml.

The contents are thoroughly mixed and allowed to stand for several minutes. Part is then centrifuged in a 15 ml. centrifuge tube. A voluminous sediment (amounting to about one-half to one-third of the total) is obtained; the upper layer consists of a slightly turbid dark brown liquid.

Exactly 1 ml. of the centrifugate (corresponding to 250 mg. yeast) is placed in a second centrifuge tube, which is well shaken while exactly 9 ml. acetone are added slowly from a burette. The tube is carefully closed with a rubber stopper, and after vigorous shaking, it is centrifuged for 3–4 min. The contents will then have separated into two layers—a very small (about 0·3 ml.) intensely coloured very viscid aqueous phase, and a clear practically colourless layer of aqueous acetone.

3 ml. (corresponding to 75 mg. yeast) of the acetone layer and 3 ml. distilled water are then mixed in a round-bottomed flask and the acetone evaporated with a water vacuum pump, without other heating than the warmth of the hand. After evaporation of the acetone, the contents are quantitatively transferred with the aid of $N/15$ KH_2PO_4 (which ensures that the reaction does not become too acid) to a graduated flask of 20 ml. capacity. The volume must not exceed 9 ml.

The colour reaction described above is carried out on the practically clear colourless solution thus obtained and, with the aid of the coefficient of extinction controlled by the standard solution (0·1 mg. of nicotinic acid), the amount of nicotinic acid contained in the sample of yeast is determined.

Comments

After treatment with NaOH, the yeast becomes a homogeneous mass; a voluminous precipitate appears on cooling and particularly on neutralizing with hydrochloric acid. The mixture is cleared by centrifuging and aliquot parts are employed for the extraction with acetone. That this is allowable is shown by the fact that the intensity of the colour produced is the same whether a part of the clear centrifugate or a part of the total mixture is used. However, it is

advisable to remove the precipitate by centrifuging; otherwise, on transference of the acetone extract to water, a turbidity will appear, which—although diminishing considerably during the colour reaction—nevertheless complicates the photometric determination. The error in the readings may amount to 5% at most; this error is avoided by removing the precipitate.

With regard to the acetone extract the following point must be mentioned: if too large an excess of acetone is used, a fairly solid resinous precipitate is formed, containing a relatively large part of the nicotinic acid (about 30%). On the other hand, too small an amount of acetone will not completely precipitate the coloured substances.

The principle to be observed in extraction is to use exactly that quantity of acetone which gives a sufficiently colourless and clear solution without leaving a precipitate that is too solid.

In the case of yeast this is achieved by using the quantities described. The precipitate amounts to about 0·3 ml., and consists partly of solid flakes, and partly of a very viscid brown liquid. Analysis of the precipitate (dissolved in 0·7 ml. distilled water and again extracted with acetone) has shown that the concentration of nicotinic acid is the same as in the acetone phase.

The transference of the acetone extract to water is rendered necessary by the fact that the presence of even small quantities of acetone during the process of heating with CNBr will result in lower values.

It is essential to evaporate the acetone at low temperature; high temperature, e.g. heating on a boiling water bath, will cause some of the nicotinic acid to volatilize with the acetone. Control analysis with known quantities have shown that the nicotinic acid is recovered quantitatively when the acetone is evaporated in vacuum with no other heating than the warmth of the hand. By evaporation on a boiling water bath of an aqueous solution of nicotinic acid (containing about 0·1 mg. of nicotinic acid) the loss is quite large; it is least when the solution contains NaOH, but very considerable if the solution is made alkaline with NH_3 or acid with a mineral acid. Presence of acetone during evaporation increases the loss of nicotinic acid.

The analysis of various kinds of yeast has given the results shown in Table II.

Table II

	Nicotinic acid in dry matter mg. %
Tuborg 140 Surface-yeast	45·5
168 Surface-yeast	44·6
113 Bottom-yeast	36·2
173 Bottom-yeast	33·9
175 Bottom-yeast	36·7
7 Wine-yeast	17·7
11 Wine-yeast	15·9
Faex medicinalis I	61·2
II	57·2
III	55·9
Compressed baker's yeast	25·7

SUMMARY

An exact colorimetric method of analysis for determining nicotinic acid in aqueous solution has been elaborated.

The method depends on the addition of cyanogen bromide at 70–80° and the production of a colour with metol (*p*-methylaminophenol sulphate) at room temperature.

The method has been employed to analyse samples of yeast. From 16 to 61 mg. of nicotinic acid % dry wt. have been found in the various types of yeast.

We wish to thank the Tuborg Breweries for submitting the yeast samples.

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XXXIII. POLYSACCHARIDES SYNTHESIZED BY MICRO-ORGANISMS

IV. THE MOLECULAR CONSTITUTION OF LUTEOSE

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(*Received 29 December 1938*)

Luteic acid, a highly mucilaginous polysaccharide, is a metabolic product of *Penicillium luteum* Zukal when this mould is grown on liquid Czapek-Dox medium with any one of the following compounds as the sole source of carbon: glucose [Raistrick & Rintoul, 1931], fructose, galactose, mannose, xylose, arabinose and glycerol [Birkinshaw & Raistrick, 1933]. Raistrick & Rintoul [1931] showed that on acid hydrolysis luteic acid yielded malonic acid (1 mol) and glucose (2 mols) and that it was probably a malonyl ester of a polyglucose. Mild alkaline hydrolysis of luteic acid eliminated the malonyl groups as malonic acid and produced a neutral polysaccharide which, when hydrolysed, yielded glucose alone, and to which the name "luteose" was given. In the present paper an investigation of the molecular structure of luteose is recorded.

The experimental work has been carried out on luteic acid produced by two different strains of *P. luteum* Zukal and the luteose samples prepared from each were examined separately. The first specimen of luteose (C. G. Anderson's preparation) was prepared from luteic acid according to the method described by Raistrick & Rintoul [1931]. It was a white powder, slightly soluble in water, mean $[\alpha]_D - 33°$. Alcohol-water fractionation revealed no essential differences in the optical properties of fractions but this fact cannot be taken as sole criterion of homogeneity. Luteose was acetylated by treatment with acetic anhydride in pyridine, followed by acetic anhydride containing sulphur dioxide and chlorine as catalysts according to Haworth & Machemer's [1932] modification of Barnett's method. Fractional precipitation of the product from chloroform solution by light petroleum gave fractions with identical optical properties and with an average acetyl content of 45·4%. The acetyl luteose was simultaneously deacetylated and methylated with dimethyl sulphate and 40% KOH in the presence of acetone by the method of Haworth *et al.* [1931]. Continued treatment raised the methoxyl content to 42·5% beyond which no increase occurred. Exhaustive extraction of this compound with acetone gave methylated luteose in 90% yield while the acetone extracts contained a methylated polysaccharide, OMe, 44·3%, which will be described later. Methylated luteose was a white powder $[\alpha]_D - 32°$, OMe, 41·5%, insoluble in acetone or ethyl alcohol. Its hydrolysis was accomplished by contact with cold fuming HCl during 156 hr. A preliminary separation of the liberated methylated sugars was made by extracting the neutralized aqueous solution with chloroform and the fractions

were separately converted into the glucosides by boiling with methyl alcoholic-HCl. The glucosides were fractionated by distillation in high vacuum and in this way it was possible to isolate 2:3:4-trimethyl methylglucoside (approx. 80%) and a dimethyl methylglucoside (15%). No tetramethyl methylglucoside was detected.

The investigation was repeated on a second sample of luteose (M. Stacey's preparation). This material was prepared from the luteic acid produced when a new strain of *P. luteum* Zukal was grown on glucose under the usual conditions. The metabolism solution was evaporated to small bulk, acidified to Congo red with HCl, the polysaccharide precipitated with alcohol and isolated in the usual way. On hydrolysis of the crude luteic acid with *N* H_2SO_4, crystalline glucose (40%), malonic acid (15%) and a syrup (45%) were obtained. The luteic acid gave less viscous solutions in water than the early specimens and, following the method of Raistrick & Rintoul, a 50% yield only of luteose was obtained. It had $[\alpha]_D - 32°$ (in sodium hydroxide) and could be separated into water-soluble and water-insoluble fractions. A reliable rapid method of isolating luteose in purified form was developed by making use of the fact that on the addition of Fehling's solution to luteic acid the malonyl groups were removed and luteose formed an insoluble copper complex. Luteose was obtained as an ash-free white granular powder, insoluble in cold water, $[\alpha]_D - 38°$ (in sodium hydroxide), and yielding only glucose on acid hydrolysis. The luteose was methylated directly with dimethyl sulphate and 30% NaOH in the presence of dioxane and recourse was not had to the intermediate formation of the acetate. Twelve methylations gave a methylated derivative having OMe, 43·6%. Attempts to increase the methoxyl content by modification of the method of methylation were unsuccessful. Methylated luteose was fractionated in the usual way and viscosity determinations on the fractions revealed its essential homogeneity.

The operations of hydrolysis of the methylated luteose with fuming HCl, conversion of the product into the glucosides and distillation of the latter, were carried out as previously described. This separation gave approximately 85% of trimethyl methylglucoside (α- and β-forms) which partially crystallized, and approximately 10% of a dimethyl methylglucoside. Despite careful search, no indications of the presence of a tetramethyl methylglucoside fraction could be obtained.

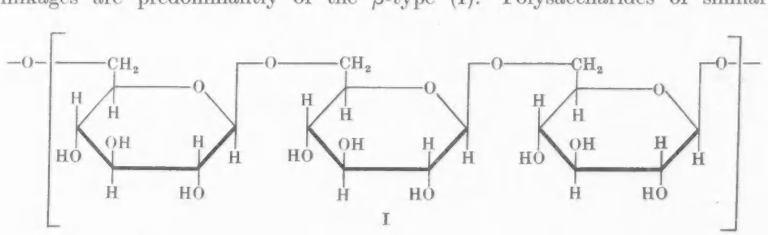
The trimethyl methylglucoside (crystalline fraction) was identified as 2:3:4-trimethyl β-methylglucopyranoside, M.P. 93–94°, since it showed no depression of M.P. in admixture with an authentic specimen and gave on hydrolysis 2:3:4-trimethyl glucose ($[\alpha]_D + 60°$ in water), identified as the anilide, M.P. 145–146°. The trimethyl methylglucoside (non-crystalline fraction) was shown to be a mixture of the α- and β-forms of 2:3:4-trimethyl methylglucopyranoside in the following way. On hydrolysis 2:3:4-trimethyl glucose ($[\alpha]_D + 60°$ in water) was isolated and yielded the anilide, M.P. 145–146° described above. 2:3:4-Trimethyl glucose was oxidized with bromine water to give 2:3:4-trimethyl δ-gluconolactone ($[\alpha]_D + 82° \rightarrow +33°$ in 26 hr.). The latter was further oxidized with HNO_3 to 2:3:4-trimethyl saccharic acid which was isolated as the lactone methyl ester (M.P. 106–107° alone or in admixture with an authentic specimen). Part of the dimethyl fraction was methylated further to form tetramethyl methylglucoside which was hydrolysed to crystalline tetramethyl glucopyranose (80% yield). The remainder was hydrolysed with 6% HCl ($[\alpha]_D^{20} + 95° \rightarrow +70°$) and a syrupy dimethyl glucose, OMe, 28·4°, $[\alpha]_D + 72°$ was isolated. This was converted into the anilide, M.P. 120–130°, OMe, 20·3%, which appeared to be a mixture and of which a satisfactory separation was not obtained.

Determination of the particle weight of methylated luteose by osmotic pressure measurements [Carter & Record, 1936] gave a value corresponding to about 80 glucose units.

Examination of the acetone extracts of methylated luteose (C. G. Anderson's preparation) revealed the presence of small amounts of a methylated polysaccharide, OMe 44·3 %, which yielded mannose derivatives on acid hydrolysis. In this connexion Anderson & Raistrick [1936] have shown that *P. luteum* Zukal, when grown on glucose under certain conditions, produced luteic acid together with small amounts of polysaccharides containing mannose, galactose or fructose units. A search was made for such compounds in the mother liquors from the purification of luteose with Fehling's solution. A polysaccharide containing galactose and mannose residues was isolated and its investigation will form the subject of a latter communication.

Discussion

The isolation of 2:3:4-trimethyl glucose in 85 % yield from methylated luteose clearly indicates that the basal constituent of the polysaccharide is a chain of glucopyranose residues united by 1:6-glucosidic linkages. Furthermore, it would appear from consideration of the optical rotation of luteose ($[\alpha]_D - 32°$) and the change in sign of rotation on hydrolysis ($\rightarrow +18°$) that the glucosidic linkages are predominantly of the β-type (I). Polysaccharides of similar



I

structure have been investigated here [Peat *et al.* 1938; Daker & Stacey, 1938] and elsewhere [Fowler *et al.* 1937]. All are of microbiological origin (the products of metabolism of the *Leuconostoc* species and of *Betabact. vermiformé* etc.) and are classed together as "dextrans". Special interest attaches to luteose in that it is the first of the dextrans investigated in which the type of linkage is β-glucosidic.

The apparent absence of an end-group (tetramethyl glucose) in methylated luteose is explicable on the assumptions either that the number of glucose units in the chain is too great to allow of the isolation of an end-group in sufficient quantity to be detectable under the conditions employed or, alternatively, that the chain exists in the form of a continuous loop. In connexion with the first hypothesis, the particle weight determined osmotically becomes of significance in that it shows the molecule to be finite in size and, indeed, not exceeding an aggregate of 80 hexose units. It is to be noted that the limits of experimental error of our method are such that the tetramethyl glucose derived from a polysaccharide constituted as an open chain of 80 units would easily have been detectable. The alternative of a closed chain structure must therefore be envisaged.

The unusually large proportion (10 %) of dimethyl glucose liberated together with the trimethyl glucose may also prove of importance when the finer structure

of the luteose molecule comes to be considered. The dimethyl glucose may arise from the presence of aggregating bonds or cross-linkages between relatively short chains of glucose units or it may simply be an expression of the observed resistance of the polysaccharide to the methylating reagents. Such resistance may be of course of a purely mechanical kind, dependent upon the physical condition of the polysaccharide during methylation and in support of this view the difficult solubility of methylated luteose in the usual solvents may be instanced. The data at present available, however, are insufficient to allow a decision to be made on this point.

Experimental

Luteose. Sample I (C. G. Anderson's preparation)

Luteic acid (112 g.) prepared from cultures of *Penicillium luteum* Zukal, Cat. No. Ad. 30 and purified according to Raistrick & Rintoul [1931] was dissolved in water (1200 ml.), *N* NaOH (178 ml.) was added, and the solution kept overnight at room temperature. The excess alkali was neutralized with *N* HCl (16·3 ml.). On standing, luteose (19 g.), $[\alpha]_D^{19°} -33°$, separated and a further sample (79 g.) was obtained by the addition of absolute alcohol up to 75%. The combined products were washed with alcohol and dried in a vacuum.

Acetylation of luteose. Luteose (75 g.) was suspended in a mixture of water (100 ml.) and pyridine (750 ml.), and to this acetic anhydride (750 ml.) was added cautiously. After being kept overnight at 15° the mixture was heated at 90° for 6 hr. The resultant gel-like solid was filtered off, washed thoroughly with pyridine and with water until free from acid and dried in a vacuum. The product was a yellow powder, acetyl content 23%. It was further acetylated with acetic anhydride and glacial acetic acid containing chlorine and sulphur dioxide and the product was isolated by pouring the reaction mixture into water. After washing with water and drying in a vacuum, acetyl luteose (70 g.) was obtained as a white powder $[\alpha]_D^{22°} -5°$ in chloroform, average acetyl content, 45·4% and on fractionation it appeared to be essentially homogeneous.

Methylation of acetyl luteose. Acetyl luteose (60 g.) was methylated with dimethyl sulphate (290 ml.) and 40% KOH (630 ml.) in the presence of acetone in the usual manner. K_2SO_4 was removed by filtration, the filtrate neutralized and evaporated to dryness in a vacuum. The residue was extracted with chloroform under reflux, the extract dried over anhydrous $MgSO_4$, and the solvent removed yielding a brown syrup (34·5 g.) OMe, 31·8%. This material was again methylated six times in a similar manner, the final product (33 g.) having OMe, 42·4%. Further methylation did not increase the methoxyl content. The methylated product (50 g.) was exhaustively extracted with acetone in a Soxhlet apparatus leaving an insoluble methylated luteose (44 g.) as a white powder, $[\alpha]_D -32°$, OMe, 41·5%. The acetone extracts containing a soluble methylated polysaccharide were retained for later investigation.

Hydrolysis of methylated luteose. The compound (35 g.) was very stable to the usual hydrolytic agents but it was satisfactorily hydrolysed by fuming HCl at 0° during 156 hr., at the end of which time a final equilibrium value $[\alpha]_D +18°$ was reached. The HCl was removed by aeration followed by addition of $BaCO_3$ in the presence of charcoal; after filtration the pale yellow filtrate and washings were combined and extracted with chloroform, the extracts dried over anhydrous $MgSO_4$ and evaporated to a syrup, fraction A_1 (8·8 g.). The aqueous mother liquor was evaporated to dryness and the residue exhaustively extracted with chloroform in a Soxhlet apparatus. Removal of the chloroform by distillation

left a syrup, fraction B_1 (24·0 g.). Fractions A_1 and B_1 were separately converted into the glucosides by heating with 1 % methyl-alcoholic HCl and these were fractionated in a high vacuum using a Widmer column. The following *main* fractions were finally obtained.

Fraction	Bath temp. 0·02 mm.	$n_D^{17°}$	Yield g.	% OMe	Remarks
		A_1. Chloroform extract (8·8 g.)			
1	130–5°	1·4573	4·24	52·1	Crystallized
2	130–5	1·4581	1·14	52·1	Crystallized
3	Residue	—	3·58	—	Crystallized
		B_1. Water-soluble material (26 g.)			
4	135–40	1·4608	15·65	—	Crystallized
5	140–50	1·4733	2·49	—	Viscous syrup
6	150–60	1·4740	2·56	—	Viscous syrup

The crystalline material, M.P. 92–93°, $[\alpha]_D - 21°$ (*c*, 1·0) in water, was drained on a tile and recrystallized from light petroleum. It was identified as 2:3:4-trimethyl β-methylglucopyranoside. No depression of M.P. was given in admixture with an authentic specimen and it was oxidized by bromine water to 2:3:4-trimethyl δ-gluconolactone, $[\alpha]_D + 82° \rightarrow + 33°$ in 26 hr. Despite careful search in the syrupy mother liquors no tetramethyl methylglucopyranoside could be detected. Fractions 5 and 6 were methylated further and hydrolysed to give tetramethyl glucose (80 % yield), M.P. 92–93°.

Luteose. Sample II (M. Stacey's preparation)

The above investigation was repeated on another sample of luteose. The luteic acid used for this sample was prepared by Mr G. Smith, in the manner previously described, from cultures of a different strain of *P. luteum*, L.S.H.T.M. Cat. No. P 211, which grows more quickly. This strain was purchased from the Centralbureau voor Schimmelcultures, Baarn, Holland, in March 1937. The metabolism solution was evaporated to a small bulk and the crude polysaccharide precipitated by addition of alcohol. This material had $[\alpha]_D - 32°$ (*c*, 1·1) ash, 5·5 %. On fractionation of this substance (5·5 g.) from water by addition of alcohol the following samples were obtained.

Fraction	Vol. of alcohol added	Yield g.	$[\alpha]_D$ corr. for ash	% ash
1 (insol. in water)	—	0·2	−32°	0·4
2	5	3·1	−34	1·2
3	8	1·0	−34	0·8
Mother liquor	—	0·4	−30	10·3

Luteic acid (5 g.) was warmed at 50° for 6 hr. with *N*/10 NaOH (100 ml.). The solution was cooled, neutralized with the exact equivalent of *N* HCl and poured into alcohol (6 vol.). The "luteose" which was precipitated was isolated (3·8 g.). It was partly soluble in cold water, $[\alpha]_D^{20°} - 32°$, and had ash content 10·0 %. The alcohol mother liquors were evaporated to a syrup, acidified with H_2SO_4 and extracted for 24 hr. with ether in a continuous extractor. Removal of the ether left a crystalline mass (1·0 g.) which was purified by sublimation and shown to be malonic acid (M.P. 133–134°). Extraction of the "luteose" (above) with cold water yielded a non-viscous soluble polysaccharide (0·6 g.), $[\alpha]_D - 25°$ (*c*, 1·2) which failed to give a precipitate with Fehling's solution in contradistinction to purified luteose which could be precipitated quantitatively as a copper luteose complex by Fehling's solution.

Luteose was prepared as follows: crude luteic acid (10 g.) was dissolved in water (200 ml.) and centrifuged, yielding an insoluble deposit (0·6 g.). The clear supernatant liquid was mixed with Fehling's solution (50 ml.) and after stirring for a few minutes a copious gelatinous precipitate separated. A further amount of Fehling's solution (100 ml.) together with distilled water (300 ml.) was added, the mixture thoroughly stirred, and kept for an hour. The precipitate was spun off, washed thoroughly with cold distilled water (all washings and supernatant liquids being retained), and dissolved in *N* acetic acid (100 ml.). Luteose was precipitated in the form of a granular powder by addition of 4 vol. of alcohol. It was separated and washed thoroughly with alcohol containing acetic acid, until completely free from copper salts, and it was finally washed with *N*/10 acetic acid and dried with absolute alcohol and ether (yield, 4·0 g.).

Luteose had the following properties: A white granular powder insoluble in cold water, soluble in sodium hydroxide, $[\alpha]_D^{20°} -38°$ (*c*, 1·1); iodine number negligible, non-reducing to Fehling's solution; could not be separated into fractions having different properties. Crystalline glucose in 95% yield was obtained on hydrolysis with $2N$ H_2SO_4 at 95°.

Isolation of a second polysaccharide from P. luteum *Zukal* (Cat. No. P. 211). The supernatant liquors from the Fehling's solution above were combined, carefully neutralized with $5N$ acetic acid and concentrated to a syrup in a vacuum. Addition of alcohol gave an impure polysaccharide in the form of a glutinous mass. It was purified by repeated precipitation from aqueous solution by alcohol and was finally obtained as a fine white hygroscopic powder, readily soluble in water, $[\alpha]_D^{20°} -25°$ (*c*, 2·4), forming non-viscous solutions. On hydrolysis with $2N$ H_2SO_4 at 95° for 2 hr. an equilibrium rotation, $[\alpha]_D^{20°} +56°$ was reached while among the products of hydrolysis crystalline *d*-galactose (66% yield) and mannose (20% yield) identified and estimated as mannose phenylhydrazone were isolated. The polysaccharide (which we propose to name "galuteose") was probably a galactan of a type similar to varianose [Haworth *et al.* 1935]. Its methylation proceeded in a manner comparable with that of varianose and the methylated product was readily soluble in acetone. Further investigations on this material will be described later.

Methylation of luteose. Luteose (10 g.) was dissolved in NaOH solution and methylated (four times) with dimethyl sulphate in the presence of acetone. The partly methylated luteose (OMe, 38%) was obtained in the form of a white powder which was insoluble in all solvents except chloroform. It was precipitated in a finely divided condition from dilute chloroform solution by addition of light petroleum and was remethylated with dimethyl sulphate and 30% NaOH in the presence of dioxane. Twelve methylations gave a product (9·2 g.), OMe, 43·8%, $[\alpha]_D^{20°} -30°$ in chloroform. Repeated attempts using different conditions were made to increase the methoxyl content but without success. Fractionation of methylated luteose (9·0 g.) was carried out as usual by precipitation from chloroform solution by light petroleum.

Fraction	Yield g.	$[\alpha]_D$ in chloroform	η_{sp}	% OMe	% ash
F_1	5·2	−31°	0·123	43·8	0·12
F_2	2·2	−29	0·100	43·6	0·16
F_3	1·5	−27	0·108	43·6	0·18

These properties provide evidence of the essential homogeneity of methylated luteose.

Hydrolysis of methylated luteose. Methylated luteose (38 g.) was hydrolysed by fuming HCl at 0° by the method described previously. The methyl glucosides

were prepared and on distillation in a high vacuum the following principal fractions were isolated.

Fraction	B.P. (bath temp.) 0·02 mm.	$n_D^{20°}$	Yield g.	% OMe	Remarks
		A_2. Chloroform extract (10·0 g.)			
1st drop	115°	1·4550	—	—	Crystallized
2nd drop	118	1·4560	—	—	,,
I	120–2	1·4560	1·1	50·9	,,
II	120–2	1·4560	4·1	50·8	,,
III	122–3	1·4560	4·0	51·0	,,
		B_2. Water-soluble material (26 g.)			
1st drop	122–3	1·4565	—	—	,,
IV (main bulk)	122–6	1·4568	22·1	50·9	,,
V	125–8	1·4569	0·5	—	,,
VI	135–45	1·4700	3·2	41·7	—

Identification of the trimethyl fractions. Fraction I (0·5 g.) was hydrolysed to the sugar which was isolated as a syrup (0·45 g.), OMe, 41·4 % (theory for trimethyl glucose 41·9 %), n_D 1·4710, $[\alpha]_D + 60°$ in water (*c*, 0·8). It was converted into the anilide [cf. Peat *et al.* 1938] which was obtained in quantitative yield, M.P. 145–146° alone or in admixture with an authentic specimen of 2:3:4-trimethyl glucopyranose anilide. No evidence of the presence of tetramethyl glucose anilide was obtained.

The crystalline fractions were combined, drained on a porous tile and recrystallized from ether-light petroleum, M.P. 93–94° alone or in admixture with a specimen of 2:3:4-trimethyl β-methylglucopyranoside. (Found: C, 50·8; H, 8·5; OMe, 52·1 %. $C_{10}H_{20}O_6$ requires C, 50·8; H, 8·5; OMe, 52·5 %.) A sample was hydrolysed in the usual way to syrupy trimethyl glucose which, on heating with the equivalent of aniline in alcoholic solution, yielded 2:3:4-trimethyl glucopyranose anilide M.P. and mixed M.P. 145–146°.

The tile was extracted with chloroform which was distilled off leaving a syrup from a portion of which 2:3:4-trimethyl glucopyranose anilide was prepared as above. A second portion was hydrolysed and the product oxidized with bromine to 2:3:4-trimethyl δ-gluconolactone, $[\alpha]_D^{21°} + 33°$ (equilibrium value, *c*, 0·9). This lactone was treated with nitric acid (sp. gr. 1·26) at 100° for 1 hr. The excess acid was removed by distillation in steam and the oxidation products converted into the methyl esters by boiling with 1 % methyl-alcoholic HCl. The main product (60 % yield) distilled at 140–145°/0·01 mm., had $n_D^{21°}$ 1·4485, crystallized on nucleation with 2:3:4-trimethyl saccharolactone methyl ester and had M.P. and mixed M.P. 106–107°.

Examination of the dimethyl fractions. Fraction VI showed $[\alpha]_D + 91°$ (*c*, 1·2 in water). A portion (0·5 g.) was methylated three times with silver oxide and methyl iodide giving a syrupy product, OMe, 61·0 %, $n_D^{22°}$ 1·4440, which was hydrolysed with HCl (6 %). The crystalline hydrolysis product (0·4 g.) isolated in the usual way was 2:3:4:6-tetramethyl glucopyranose, M.P. 84° alone or in admixture with an authentic specimen. A further portion (1·0 g.) was hydrolysed with HCl (6 %), $[\alpha]_D^{20°} + 95°$ changing to $[\alpha]_D^{20°} + 70°$ (equilibrium value) in 6 hr. The syrupy product (0·6 g.) had $[\alpha]_D + 72°$ in water, OMe, 28·4 % (dimethyl glucose requires OMe, 33·6 %). Under the usual conditions this product yielded an anilide in 20 % yield. This had M.P. 120–130°, and was apparently a mixture. Its separation by fractional crystallization was not satisfactorily accomplished and the exact configuration of the dimethyl fraction is under investigation.

Molecular weight of luteose

Osmotic pressure measurements to determine the molecular weight were carried out by Mr W. T. Chambers to whom we are greatly indebted. A value corresponding to 84 glucose units was obtained.

SUMMARY

Luteose, the neutral polysaccharide produced by elimination of the malonyl residues from luteic acid, a metabolic product of *Penicillium luteum* Zukal, has been shown to be constituted mainly of β-glucose units linked through the 1:6-positions. The molecule may be a terminated linear chain but determination of its molecular weight (84 units) by osmotic pressure measurements and the presence of dimethyl glucose (10%) among the products of hydrolysis of methylated luteose, indicates that the molecule is more likely to be of the closed chain type.

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XXXIV. ORGANIC ANIONS OF HUMAN ERYTHROCYTES

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(*Received 23 December 1938*)

In their efforts to simplify the two phase physico-chemical system of the blood, Henderson [1928] and other authors [Van Slyke *et al.* 1923; Dill *et al.* 1937] assumed that anions in normal human erythrocytes other than Cl^-, HCO_3^- and Hb^- (designated by Henderson X^-) were usually insignificant in amount. This assumption was supported by analyses which seemed to show that any excess of base over $Cl^- + HCO_3^- + Hb^-$ was negligible. It is now clear, however, that the values assigned to Hb^- were too high either because it was assumed that the buffering powers of human and horse haemoglobins were similar or because the data were derived from haemolysed erythrocyte solutions instead of from solutions of pure Hb. Maizels & Paterson applied Adair's [1925] titration curves to the data of Henderson [1928] and of Dill *et al.* [1937], and found that the base was actually greater than $Cl^- + HCO_3^- + Hb^-$; they confirmed these observations [1937] experimentally by analysing a series of erythrocyte solutions and calculating Hb^- from their own electrometric titrations of Hb.

It thus became necessary to assume the presence of another anion which, following Henderson, they called X^-, and which further appeared to be roughly inversely proportional to the Hb content per litre of original cells, being much greater in the haemoglobin-deficient cells of certain anaemias than in normal erythrocytes. In the latter, X^- was about equal to Hb^-, while in anaemic cells it might be twice as great as Hb^-. Further, knowing the Hb content per 1000 ml. cells it was found possible to foretell the probable value of X^- to within $\pm 15\%$ (Fig. 1).

It will be realized that the amount of X^- depends ultimately on the titration curve of Hb^- and although the figures found by Adair [1925] and by Maizels & Paterson [1937] are probably correct, in order to remove any ambiguity on this score an effort was made to demonstrate the presence of X^- in the absence of Hb by analysing the dialysate of laked cell solutions. A number of experiments were done but as the fuller and more satisfactory experiments carried out subsequently are described later in this paper, the results of the original experiments will only be summarized here.

The dialysate of laked cell solutions contained a marked excess of base over Cl^- and HCO_3^-, so that the presence of X^-, apparently responsible for 30% of total base-binding, in dialysates was confirmed. The titration curve of the dialysate showed marked buffering between *p*H 6 and 7 and in this resembled the titration curve of phosphate and the phosphoric esters [Meyerhof & Suranyi, 1926] but while the buffering power of phosphate greatly decreases at $pH > 7$, the buffering power of the dialysate was maintained until well beyond *p*H 9 and this part of the curve recalled the titration curve of glutathione [Pirie & Pinhey, 1929]. Analysis of the dialysates showed in fact that organic phosphorus and SH— groups were present. It therefore seemed reasonable to assume that phosphoric esters and glutathione might account in part at least for X^-. Support for this view was forthcoming from results already available in the literature

which may be summarized as follows: human erythrocytes contain about 70 mg./100 ml. glutathione [Platt, 1931] which would bind 1·1 equiv. base per mol. at the *p*H of the erythrocyte (7·5 at 20°), and about 50 mg./100 ml. acid-soluble P [Kay *et al.* 1927; Kerr & Daoud, 1935; Rapoport, 1937] which binds 2·4 equiv. base per atom P at *p*H 7·5 [Farmer & Maizels, 1938]; approximately 25% is easily hydrolysable phosphate, probably pyrophosphate corresponding in whole or in part to adenosine triphosphate [Kerr & Daoud, 1935], which binds 1·3 equiv. base per atom P, while the residue consists of inorganic phosphate, hexosephosphate and other phosphates binding little <2 equiv. base per atom P at *p*H 7·5. At a conservative estimate then cell P should bind 1·9 equiv. base per atom P so that theoretically cell P and glutathione should bind together 32 m. equiv. base per 1000 ml. cells.

Since this is about twice as great as the amount of base bound by Hb, it is extremely remarkable that workers on the acid-base equilibrium of the blood should hardly have taken the base-binding properties of phosphoric esters into account at all. Comments have indeed been made on the compensatory decrease in cell P which occurs in experimental acidosis [Haldane *et al.* 1924; Kay, 1924; Rapoport, 1937], but the only direct reference we have found is by Peters & Van Slyke [1931] who, in contrast with Van Slyke's statements of 1923, were able to say in 1931: "it has generally been assumed that proteins are the chief

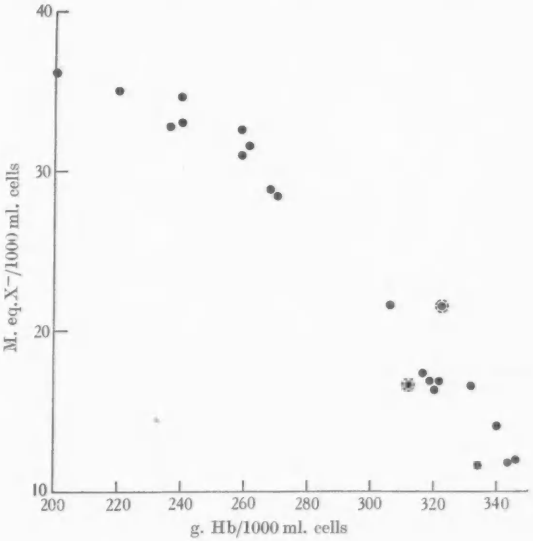

Fig. 1.

buffers in cells; but it is quite possible that organic phosphates are more important". We shall see later that this suggestion is incorrect since, the importance of the organic phosphates lies primarily in their base-binding and not in their buffering powers. In view of the amount of base that could theoretically be bound by the acid-soluble P of cells, we have ourselves carried out a number of new analyses on normal and anaemic cells and have compared

the theoretical amount of base bound by cell phosphate and glutathione with the amount probably bound by X^-, the non-haemoglobin organic anions of erythrocytes, as determined from Fig. 1.

Methods

Erythrocytes were separated for analysis by centrifuging blood which had been treated with phosphate-free heparin (Hoffmann-la Roche). Whole blood was kept on ice as far as possible, and despite the delay in adding trichloroacetic acid Table I shows that little hydrolysis of phosphoric esters occurred. Inorganic P and total acid-soluble P were determined according to Martland & Robison [1926]. Easily hydrolysable P was estimated by boiling the trichloroacetic acid filtrate for 7 min. with N H_2SO_4, while the glutathione was estimated iodimetrically in acid solution [Platt, 1931]. The results are summarized in Table I and are expressed in mg. or m. equiv. per 1000 ml. cells.

Table I

Case	Hb g. blood	Hb g. cells	Total acid-sol. P mg.	Hydro-lysable P mg.	Inor-ganic P mg.	Gluta-thione (Gl) mg.	P+Gl m. equiv.	X^- m. equiv.
1. Normal	156	335	485	93	17·4	680	32	14
2. Normal	148	333	465	125	12·1	810	31·5	16
3. Hyperpiesis	118	325	550	94	30·5	790	36·5	17
4. Normal	124	320	527	124	21·4	600	34	18
5. Normal	152	318	510	—	20·7	740	34	19
6. Hyperpiesis	114	312	510	—	20·1	730	33·5	21
7. Normal	150	312	540	115	18·8	980	36·5	21
8. Normal	146	308	462	103	16·9	800	31	21
9. Normal	136	302	510	122	23·0	840	34	23
10. Prostatectomy	141	301	457	100	17·0	970	31·5	23
11. Microcytic anaemia	88	280	680	115	22·5	1040	45·5	28
12. Microcytic anaemia	59	260	592	114	18·1	910	39·5	31
13. Microcytic anaemia	70	259	691	128	22·6	1050	46	31
14. Microcytic anaemia	70	230	702	130	22·3	1140	47	35
15. Microcytic anaemia	52	229	675	—	17·0	1130	45·5	35
16. Microcytic anaemia	36	203	625	118	16·1	1350	43	38
17*a*. Macrocytic anaemia	50	283	610	110	15·2	1130	41·5	?
17*b*. Macrocytic anaemia	63	267	692	100	19·2	1220	46·5	?
18. Acholuric jaundice	134	343	450	106	21·5	600	29·5	10

The cases fall into three groups: 10 without anaemia, 6 with hypochromic microcytic anaemia and 1 with macrocytic anaemia. The two main groups are placed in order of their Hb content per 1000 ml. cells. The analyses of normal cells correspond with those of other workers; in microcytic anaemia both acid-soluble P and glutathione are increased but the correlation between these increases and Hb either in unit volume of cells or whole blood is not close. The possibility of increases in P and glutathione being associated with reticulocytosis was considered but since reticulocytosis in the first 16 cases never exceeded 2% this could not have been a cause. Again, in case 17, 0·25% of reticulocytes were present at the first examination (*a*) and 25% at the second (*b*), but glutathione was only increased by 10%. Finally, the last case in the series had 4·8% reticulocytes but total P and glutathione were the lowest recorded. At present the relation of high glutathione values to anaemia therefore remains obscure. The function of the increased phosphoric esters is a little more clear; in microcytic anaemia Hb and base bound by Hb decrease while cell water increases; phosphoric esters will help to maintain base-binding and, being osmotically active, will support the osmotic pressure of the cell contents. On the other hand, phosphates are not good buffers at pH > 7 and they probably play little part in

cell buffering in normal or anaemic cells: assuming that 1000 ml. normal cells contain 320 g. Hb binding 20 m. equiv. base at *p*H 7·25, and that there are also present 500 mg. acid-soluble P binding 30 m. equiv. base at *p*H 7·25, then it would require 4 m. equiv. base to shift a solution of the Hb alone to *p*H 7·35 and only 0·6 m. equiv. to effect a corresponding change in the phosphoric esters alone. In anaemia on the other hand the buffering power of the phosphoric esters is a little more significant.

If cell phosphate bound its full theoretical amount of base at cell *p*H it would combine with nearly twice as much base as Hb itself. Moreover, since plasma contains very little phosphoric ester, the acid-soluble phosphates within the erythrocyte should act as non-permeating anions whose effect on the distribution of permeating ions should far outweigh that of haemoglobin. For this reason equations dealing with the distribution of water and electrolytes between cells and plasma, which refer solely to Hb as the major non-permeating ion are likely to have no more than comparative significance.

Base bound by X^- and the phosphoric esters

If the phosphoric esters and glutathione in the cells were to bind their full theoretical quota of base, then X^-, that is $B^+ - Cl^- - HCO_3^- - Hb^-$, should be equal to or greater than the equivalence of the phosphoric esters + glutathione. Actually, comparison of the last two columns in Table I shows that the reverse is the case and this can only arise in one of four ways: (*a*) less than 1·9 equiv. base is bound per atom P; (*b*) an unidentified excess of base is present in the erythrocytes; (*c*) the value of $B^+ - Cl^- - HCO_3^- - Hb^-$ is too small owing to some analytical error; (*d*) phosphoric esters and/or chloride exist in some form in which they fail to bind base. Consideration of these possibilities leads to the following conclusions. (*a*) It is probable that P does in fact bind at least 1·9 m. equiv. base per atom at cell *p*H. This was confirmed in the following way: a trichloroacetic acid filtrate of erythrocytes was neutralized with ammonia using methyl orange as an indicator. Basic lead acetate and alcohol were added to precipitate the phosphates. The deposit was well washed, first with dilute ammonia solution to remove acetate, trichloroacetate, Na and K, and then with alcohol and water to remove ammonia. Lead was removed with H_2S and the filtrate evaporated *in vacuo* at 40°. The residue was taken up in water and found to be free from ammonia and sulphur. The P content of this solution was estimated and it was then titrated electrometrically at 38°. It was found that each atom of P corresponded to 1·86 equiv. base at *p*H 7 and 2·03 equiv. at *p*H 7·5. (*b*) There is no evidence of the presence of any unidentified excess of base, but if such were present, then it should be recovered by electrodialysis. (*c*) The values for base and chloride recorded by Maizels & Paterson [1937] were obtained by well-tried methods which gave consistent results agreeing with the figures of other workers. Of these, the data of Dill *et al.* [1937] may be quoted. These workers, using entirely different methods, give the following average figures of twelve individuals: base, 11·0; Cl, 5·6; HCO_3, 1·75 m. equiv. per 100 ml., whence $X^- + Hb^- = 3{\cdot}6$ m. equiv./100 ml.

If Hb^- is calculated according to the data of Dill *et al.*, $X^- = -0{\cdot}07$ m. equiv., but employing the more accurate data of Adair [1925] or of Maizels & Paterson [1937] for the buffering power of reduced Hb, then Hb^- may be calculated as follows:

$$Hb^- = Hb \times (pH - \text{I.P.}) \times B_r$$

where Hb equals the amounts of haemoglobin present in g.; *p*H its *p*H; I.P. the isoelectric point of reduced Hb (6·81 at 38°) and B_r the buffering power of

reduced Hb per g., which is 0·138 m. equiv. per pH unit between 6·8 and 7·3. Then in the experiments of Dill *et al.*, $Hb^- = 33{\cdot}5 \times (7{\cdot}167 - 6{\cdot}81) \times 0{\cdot}138 = 1{\cdot}7$ m. equiv. per 100 ml. cells, and $X^- = 3{\cdot}6 - 1{\cdot}7 = 1{\cdot}9$ m. equiv. per 100 ml. blood. This agrees well with the corresponding figure given by Maizels & Paterson, and there is therefore no reason to ascribe the apparent excess of base to analytical error. (*d*) There remains the fourth possibility that phosphoric esters and/or chloride within the cell bind less base than they do in simple aqueous solution. This might possibly occur through some form of complex association and at present represents the most likely explanation; experimental evidence on the point is however so far lacking. One further point in this connexion does indeed emerge from Table I: although X^- and acid-soluble phosphate are both increased in hypochromic microcytic anaemia, the increase in X^- is relatively the greater and this suggests the possibility that the increase in X^- may be accomplished by the diversion of phosphate from a complex to a simple form.

In the attempt to obtain direct evidence as to the state of phosphoric esters within the cell, laked cell solutions were dialysed with the object of determining whether X^- and the phosphoric esters dialysed at the same rate and whether the apparent excess of total phosphate over X^- was to be observed in the dialysate as well as in the dialysed solutions.

X^- and phosphoric esters in dialysed cell solution and dialysate

Human erythrocytes were washed 5 times with isotonic KCl solution, packed by centrifuging and the supernatant KCl removed with a capillary pipette. The cells were then mixed with half their volume of distilled water, vigorously shaken with a similar volume of ether and recentrifuged. A compact ethereal scum rose to the surface, containing lipins, cell stromata and other substances. The subjacent clear laked cell solution was removed and freed from ether by evaporation *in vacuo* at room temperature; it was then dialysed in cellophane bags against one-third its volume of water, the systems being mixed by inversion every 2 hr. Dialysis was carried out in two cases for 18 hr. and in one case for a week. In no case was true equilibrium attained. Finally, the dialysed cell solution and dialysate were analysed. Details of the methods employed are given in the appendix together with a full statement of all the data obtained in Exp. 2. The results of all three experiments are summarized in Table II, where the following are set out:

(*a*) No. of hr. of dialysis; (*b*) vol. of cell solution and of dialysate; (*c*) Hb in g. per 1000 ml.; (*d*) electrometric measurement of pH; (*e*) Hb^- in m. equiv. per 1000 ml. $Hb \times (p\text{H} - \text{I.P.}) \times B_r$; (*f*) Cl in m. equiv. per 1000 ml.; (*g*) HCO_3^- in m. equiv. per 1000 ml.; (*h*) base bound by glutathione (Gl^-) per 1000 ml. at the experimental pH (calculated from the titration curves of Pirie & Pinhey [1929]); (*i*) total base (this is one of the most crucial as well as the most difficult estimations; it was carried out by three different methods: (i) electrolytically; (ii) as the sum of $K + Na + Mg + NH_4$; (iii) as sulphate by the benzidine precipitation method; (*j*) X^- in m. equiv. per 1000 ml. (in this case,

$$X^- = Base^+ - Cl^- - HCO_3^- - Hb^- - Gl;$$

CO_2 and glutathione are quite small and may well be neglected; they are, however, included for the sake of completeness); (*k*) inorganic phosphate; (*l*) total acid-soluble P mg. per 1000 ml.; (*m*) total acid-soluble phosphate in m. equiv. per 1000 ml.; it has been assumed that 1 atom acid-soluble P binds 1·9 m. equiv. base at pH 7·2; if all cell phosphate is fully ionized then base bound by X^- should be as great as that bound by total phosphate or greater.

Table II. T. = 38°

	Exp. 1		Exp. 2		Exp. 3	
	Cell soln.	Dialysate	Cell soln.	Dialysate	Cell soln.	Dialysate
No. of hr. dialysis	18		18		168	
Vol. ml.	50·4	34	53	38	63·5	33
Hb colorimetric g./1000 ml.	11·4	0	17·7	0	13·4	0
Hb gasometric g./1000 ml.	—	—	18·1	—	—	—
*p*H	7·29	7·68	7·19	7·65	7·19	7·57
Hb^- m. equiv./1000 ml.	7·5	0	9·7	0	7·0	0
Cl^- m. equiv./1000 ml.	21·2 ±0·5	25·8 ±0·4	33·1 ±0·2	40·2 ±0·2	32·3 ±0·5	37·5 ±0·5
HCO_3^- m. equiv./1000 ml.	0·2	0·14	0·2	0·1	0·1	0·1
Gl^- m. equiv./1000 ml.	0·64	0·85	0·58	0·66	0·57	0·67
Total base:						
Electrolytic m. equiv./1000 ml.	32·1 ±0·4	31·9 ±0·2	48·4 ±0·6	47·5 ±0·5	48·1 ±0·5	52· ±1·0
K + Na + Mg + NH_4 m. equiv./1000 ml.	—	—	48·0 ±1·0	47·6 ±0·2	—	—
as SO_4 m. equiv./1000 ml.	—	—	48·4 ±1·2	—	—	—
X^- m. equiv./1000 ml.	2·6 ±1·0	5·1 ±0·6	4·7 ±1·2	6·4 ±0·7	8·1 ±1·0	13·8 ±1·5
P inorganic mg./1000 ml.	24·7	12·2	15·0	6·9	59·5	54·3
P acid-sol. mg./1000 ml.	144·3 ±1·3	70·1 ±0·9	224 ±1·0	79·9 ±0·9	185 ±3·0	140 ±1·5
Total acid-sol. phosphate —$RHPO_4^-$ m. equiv./1000 ml.	8·8 ±0·1	4·3 ±0·06	13·7 ±0·06	4·9 ±0·06	11·3 ±0·2	8·6 ±0·1
X^-/—$RHPO_4^-$	0·3	1·2	0·34	1·3	0·7	1·6

The following conclusions may be drawn from Table II.

(1) The dialysate is much more alkaline than the dialysed cell solution; this is to be expected since the cell solution contains the non-penetrating anions Hb^- and organic phosphates.

(2) Chloride, glutathione, base and X^- diffuse rapidly.

(3) Acid-soluble phosphate diffuses slowly through cellophane; at 18 hr. the dialysate contains less than one-half the amount of acid-soluble P present in the cell solution, while after 7 days' dialysis, P in the dialysate is only two-thirds of that in the cell solution. It will also be noted that the ratio of inorganic to total acid-soluble P rises as the period of dialysis increases and this is doubtless because hydrolysis by cell phosphatases is prolonged.

(4) When all allowance is made for experimental errors, it is still true that after 18 hr. dialysis, the theoretical amount of base that could be bound by total phosphate in the cell solution is twice as great as that bound by X^-, while in the dialysate it is slightly less. The observations gain strength from the fact that the same methods, often in quadruplicate, were used on both solutions which differed only in that one contained haemoglobin while the other did not. Further, of all the values given in Table II, there is only one assumed figure and that is the *p*H at which reduced haemoglobin is isoelectric. This is usually taken to be 6·81; if in fact the isoelectric point were lower, then the ratio of X^- to phosphate would be even less; if on the other hand the isoelectric point were higher, then X^- and phosphate would tend to become more nearly equal, though X^- would still be much less than phosphate unless the isoelectric point of haemoglobin at 38° were at *p*H > 7. Rejecting the latter remote possibility, one is forced to conclude that at least half of the phosphate in cells is not binding base but is complexly combined. The only possible alternative to this view is the unlikely one that chloride and not phosphate is complexly combined.

Although the systems are not in equilibrium, it is still of interest to compare the concentrations of ions in the cell solution and in the dialysate. Concentrations are obtained by dividing the known ion contents by the amount of water present

in the corresponding phase. In the case of cell solutions a figure has been subtracted from the measured total content of water equal to 0·2 ml. bound water per g. Hb (Table III).

Table III

	Exp. 1	Exp. 2	Exp. 3
$[H_2O]$ cell soln./$[H_2O]$ dialysate	0·85	0·86	0·85
$[H^+]$ cell soln./$[H^+]$ dialysate	2·45	2·82	2·40
[Base] cell soln./[Base] dialysate	1·19	1·18	1·09
$[Cl^-]$ dialysate/$[Cl^-]$ cell soln.	1·04	1·05	1·00
[Gl] dialysate/[Gl] cell soln.	1·12	0·98	1·00
$\sqrt{}$[Acid-sol. P] dialysate/$\sqrt{}$[Acid-sol. P] cell soln.	0·64	0·56	0·80
$[X^-]$ dialysate/$[X^-]$ cell soln. and $\sqrt{}[X^-]$ dialysate/$\sqrt{}[X^-]$ cell soln.	1·7 (1·3)	1·2 (1·1)	1·4 (1·2)

The phosphate ratio has been calculated on the assumption that P is divalent and the X^- ratio is shown both for a univalent and for a divalent anion.

It will be seen that the dialysate is much more alkaline than the cell solution and this has already been attributed to the excess of absolutely or relatively non-permeating anions, Hb and phosphate in the cell solution. But while $[H^+]$ ratios are similar in all three experiments, the phosphate ratios are very different, for in Exps. 1 and 2, internal phosphate is twice external phosphate, while in Exp. 3, after a whole week's dialysis total acid-soluble phosphates within and without the cellophane bag are much more nearly equal. Hence changes in phosphate distribution are not accompanied by changes in the distribution of H^+, which suggests that phosphate in whole or in part is not acting as a relatively non-permeating anion and is in fact not acting as an anion at all. For, were phosphate in the cell solution acting as an anion a decrease in the phosphate ratio would bring about a decrease in the *p*H difference between cell solution and dialysate.

It will further be noted that $[Cl^-]$ is greater in the dialysate and $[B^+]$ greater in the cell solution; this is doubtless brought about by the excess of Hb^- in the cell solution. While $[B^+]$ cell solution/$[B^+]$ dialysate and $[Cl^-]$ dialysate/$[Cl^-]$ cell solution are in rough conformity, neither ratio agrees at all with the $[H^+]$ ratio. Apart from the fact that all the systems are still far from equilibrium, there seems no explanation for this discrepancy. Phosphate has of course diffused very slowly and even after a week's dialysis is still greater in the cell solution.

Finally, we may note that while the dialysate/cell solution ratios of Cl, glutathione and X^- on the one hand, and the cell solution/dialysate ratio of base on the other, are in rough agreement, the phosphate ratios show no correspondence at all. Since the correspondence of $[Cl^-]$ ratios is based on the assumption that Cl^- in cell solution is fully ionized, it is reasonable to believe that this assumption is correct; the phosphate ratios alone disagree with the other ion concentration ratios, while if one assumes that chloride and not phosphate in the laked cell solution is complexly combined, then both the phosphate and chloride ratios would be out of keeping with the ratio of base concentration.

Summary

In normal human erythrocytes, $Base^+$ is equal to the sum of $Cl^- + HCO_3^- + Hb^-$ together with certain other complex organic anions symbolized by X^-. X^- binds about as much base as Hb^-, i.e. about 20 m. equiv. per 1000 ml. cells. Since erythrocytes contain phosphate (organic and inorganic) and glutathione, it is reasonable to assume that these substances may account for at least a part of X^-. Cell phosphate consists of diphosphoglycerate (65 %), adenosine triphosphate

(25%) and other phosphates. These compounds in aqueous solution at *p*H 7·5 bind at least 1·9 m. equiv. base per atom of P. Since erythrocytes contain 500 mg. acid-soluble P per 1000 ml., this quantity of phosphate should bind 30 m. equiv. base, a quantity far in excess of X^-. It would therefore appear that much of the acid-soluble phosphate in cells is not ionized but is complexly combined.

If erythrocytes are dialysed, X^- is slightly greater than acid-soluble phosphate in the dialysate, but is very much less than acid-soluble phosphate in the dialysed cell solution. This shows that acid-soluble phosphate is fully ionized in the dialysate, but is only partially ionized in the laked cell solution.

In the erythrocyte of microcytic hypochromic anaemia, haemoglobin is decreased while X^-, glutathione, acid-soluble phosphate and water are all raised; this helps to maintain the base-binding and osmotic pressure of the anaemic cell. The function of cell phosphate as a buffer at the *p*H of the normal cell is relatively insignificant; in the anaemic cell at the same *p*H, it may be slightly more important.

Appendix

Methods

Haemoglobin. (*a*) Colorimetric, by Haldane's haemoglobinometer calibrated against a gasometrically standardized solution of blood. Duplicates should agree within ±2%.

(*b*) Manometric [Peters & Van Slyke, 1931].

*p*H. This was measured in the hydrogen electrode after the method of Harington & Neuberger [1936]; the standardizing solution was 0·01 *N* HCl in 0·09 *N* NaCl and the values were sometimes confirmed with an acetate buffer. With these standard solutions reproducible E.M.F. were always obtained.

Chloride [Claudius, 1924]. Wet ashing is accomplished by boiling with HNO_3 in the presence of $KMnO_4$ as an oxidizing agent. The results obtained by this method have been compared with those obtained when H_2O_2 is substituted as an oxidizing agent and also with the results obtained on a protein-free filtrate of cell solution treated with sodium tungstate and sulphuric acid, where chloride is estimated without a preliminary wet ashing. The agreement between all three methods was very close.

Glutathione [Platt, 1931]. Glutathione is estimated by iodimetric titration in acid solution. The material is first reduced with zinc and hydrochloric acid.

CO_2—manometric.

Total base [Adair & Keys, 1934]. (*a*) At Dr Adair's suggestion we have used cellophane instead of collodion to close the cathode tube, and have found it best to electrodialyse for a first period of 24 hr., and titrate. We have found it advantageous to replace the titrated sulphuric acid by fresh acid and then electrodialyse for a further 24 hr., after which the second lot of acid is titrated. The first electrodialysis recovers about 95% of the base, and the second, the rest.

(*b*) Total base as $K + Na + Mg + NH_4$: Potassium was estimated by the method of Kramer & Tisdall [1921]; sodium, after Salit [1932]; magnesium by the technique described by Briggs [1924]. The test adopted for ammonia was nesslerization of a tungstate—sulphuric filtrate: it was negative in all cell solutions.

(*c*) Total base as sulphate, both directly [Fiske, 1922] and indirectly [Stadie & Ross, 1925].

Phosphorus was measured by the methods described by Martland & Robison [1926].

Protocol of Exp. 2

Hb. Colorimetric. 176 and 178 g. per 1000 ml.
Gasometric. 181 g. per 1000 ml.

pH. Standard solution: 0·01 *N* HCl in 0·09 *N* NaCl.
E.M.F. standard HCl + calomel half cell = 363 mV. at 38°.
E.M.F. HCl = 2·08 × 61·7 = 128 mV.
E.M.F. calomel half cell = 235 mV.
E.M.F. cell solution = (*a*) 678·5; (*b*) 678·5 mV. *p*H = 7·19.
E.M.F. dialysate = 707 mV. *p*H = 7·65.

Chloride. Solutions used: 0·5 ml. $N/40$ $AgNO_3$. 2 ml. of 2 in 15 dilution of cell solution or dialysate.

$AgNO_3$ = 1·200; 1·200; 1·205; 1·200; 1·200 ml. $N/96{\cdot}2$ KCNS.
$AgNO_3$ + cell solution = 0·35; 0·35; 0·36; 0·36; 0·36; 0·36 ml. $N/96{\cdot}2$ KCNS.
$AgNO_3$ + dialysate = 0·17; 0·16; 0·17; 0·16; 0·165; 0·16 ml. $N/96{\cdot}2$ KCNS.
Chloride m. equiv. per 1000 ml. cell solution = $33{\cdot}1 \pm 0{\cdot}2$.
Chloride m. equiv. per 1000 ml. dialysate = $40{\cdot}2 \pm 0{\cdot}2$.

HCO_3. Cell solution: CO_2 = 0·4 vols. % = 0·2 m. equiv. per 1000 ml.
Dialysate: CO_2 = 0·18 vols. % = 0·1 m. equiv. per 1000 ml.

Glutathione. Cell solution: 2 ml. of 1 in 2 dilution in trichloroacetic acid
= 0·58 ml. $N/1100$ I_2 = 0·53 ml. $N/1000$ I_2.
1000 ml. cell solution contain: 0·53 m. mol. = 0·58 m. equiv. glutathione at *p*H 7·2.
Dialysate. 1 ml. = 0·61 ml. $N/1100$ I_2 = 0·55 ml. $N/1000$ I_2
= 0·55 m. mol. = 0·66 m. equiv. glutathione at *p*H 7·65.

Total base electrolytic. Solutions: 1 ml. $N/100$ H_2SO_4. 1 ml. of 2 in 15 dilution of cell solution or dialysate.

1 ml. $N/100$ H_2SO_4 = 1·000; 1·002; 1·004; 1·002 ml. $N/100$ NaOH.
H_2SO_4 + cell solution = 0·365; 0·359; 0·351; 0·350 ml. $N/100$ NaOH.
H_2SO_4 + dialysate = 0·366; 0·374; 0·364 ml. $N/100$ NaOH.
Base m. equiv./1000 ml. cell solution = $48{\cdot}4 \pm 0{\cdot}6$.
Base m. equiv./1000 ml. cell solution = $47{\cdot}5 \pm 0{\cdot}5$.

Total base as K + Na + Mg + NH_4. K m. equiv./1000 ml. cell solution
= 41·2; 41·0; 41·2; 41·0; 41·0
= $41{\cdot}1 \pm 0{\cdot}1$.
K m. equiv./1000 ml. dialysate = 43; 43·1; 43; 43. = 43 ± 0.
To these must be added $\pm 0{\cdot}2$ corresponding to variations in the standard solutions
K m. equiv./1000 ml. cell solution = $41{\cdot}1 \pm 0{\cdot}3$.
K m. equiv./1000 ml. cell solution = $43{\cdot}0 \pm 0{\cdot}2$.

Sodium. Na m. equiv./1000 ml. cell solution = 3·7; 4·4. = $4{\cdot}05 \pm 0{\cdot}3$.
Na m. equiv./1000 ml. dialysate = 3·2; 3·2. = $3{\cdot}2 \pm 0$.

Magnesium. Mg. m. equiv./1000 ml. cell solution = 3·1; 2·5 = $2{\cdot}8 \pm 0{\cdot}3$.
Mg. m. equiv./1000 ml. dialysate = 1·4.

Ammonium. Absent in cell solution and dialysate.

Total base as sulphate.
Direct: Cell solution m. equiv./1000 ml. = 47·2; 48·3; 48·7; 47·9.
Indirect: Cell solution m. equiv./1000 ml. = 49·4; 47·5; 49·6; 48·4.
Total base as sulphate in cell solution
m. equiv./1000 ml. = $48{\cdot}4 \pm 1{\cdot}2$.

Total acid soluble phosphorus. Three equal standards agreed exactly.
Cell solution mg./1000 ml. = 225; 225; 224; 225; 223.
Dialysate mg./1000 ml. = 80; 80·8; 79; 80·2.

One of us (S. N. F.) is indebted to the Surgical and Medical Units of University College Hospital for a personal grant, and the other (M. M.) to the Graham Research Fund for a grant for the purchase of apparatus.

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